

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE FORM PTO-1390 (REV. 12-2001)		ATTORNEY'S DOCKET NUMBER <b>RUT.00-0038US</b> U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>not yet assigned 10/088634</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		
INTERNATIONAL APPLICATION NO. <b>PCT/US00/25939</b>	INTERNATIONAL FILING DATE <b>21 September 2000</b>	PRIORITY DATE CLAIMED <b>21 September 1999</b>
<b>TITLE OF INVENTION</b> <b>SITE-SPECIFIC RECOMBINATION SYSTEM TO MANIPULATE THE PLASTID GENOME OF HIGHER PLANTS</b>		
<b>APPLICANT(S) FOR DO/EO/US</b> <b>MALIGA, Pa1; CORNEILLE, Sylvie; LUTZ, Kerry</b>		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li><input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</li> <li><input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</li> <li><input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto.</li> <li>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</li> </ol> </li> <li><input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input type="checkbox"/> have been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li><input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li><input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li><input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>		
<b>Items 11 to 20 below concern document(s) or information included:</b>		
<ol style="list-style-type: none"> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li><input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</li> <li><input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li><input type="checkbox"/> A substitute specification.</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li><input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</li> <li><input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</li> <li><input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</li> <li><input checked="" type="checkbox"/> Other items or information: <b>Copy of Form PCT/IB/308</b></li> </ol>		

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER		
<u>Int'l App 088634</u>	PCT/US00/25930	RUT.00-0038US		
<p>21. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p><b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b></p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1040.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$890.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$740.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$710.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00</p>		CALCULATIONS PTO USE ONLY		
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		\$ 100.00		
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$		
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$
Total claims	21 - 20 =	1	x \$18.00	\$ 18.00
Independent claims	2 - 3 =	0	x \$84.00	\$ 0
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$280.00	\$ 0
<b>TOTAL OF ABOVE CALCULATIONS =</b>		\$ 118.00		
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		+ \$ 59.00		
<b>SUBTOTAL =</b>		\$ 59.00		
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		\$ 0		
<b>TOTAL NATIONAL FEE =</b>		\$ 59.00		
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +		\$ 0		
<b>TOTAL FEES ENCLOSED =</b>		\$ 59.00		
		Amount to be refunded:	\$	
		charged:	\$	
<p>a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>59.00</u> to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>04-1406</u>. A duplicate copy of this sheet is enclosed.</p> <p>d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.</p>				
<p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.</b></p>				
<p>SEND ALL CORRESPONDENCE TO:</p> <p>Kathleen D. Rigaut, Ph.D., J.D. DANN, DORMAN, HERRELL AND SKILLMAN 1601 Market Street, Suite 720 Philadelphia, Pennsylvania 19103 United States of America</p>				
 SIGNATURE <u>Kathleen D. Rigaut, Ph.D., J.D.</u> NAME <u>43,047</u> REGISTRATION NUMBER				

JC13 Rec'd PCT/PTO 20 MAR 2002

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re the Application of )  
Pal Maliga et al. )  
Serial No. Not yet assigned )  
[Int. Appl. No. PCT/US00/25930] )  
Filed: September 21, 2000 )  
For: "Site-specific )  
Recombination System to )  
Manipulate the Plastid )  
Genome of Higher Plants")

**PRELIMINARY AMENDMENT**

The present application is based on International Application PCT/US00/25930. Before calculation of the filing fee, please amend the above-referenced patent application as follows:

**In the specification:**

At page 1, line 5 of the specification, please insert the following priority claim:

-- This application is a §371 filing of PCT/US00/25930 filed September 21, 2000 which in turn claims priority to US provisional applications 60/155,007 and 60/211,139 filed September 21, 1999 and June 13, 2000, respectively, each of which is incorporated by reference herein.--

At page 60 of the application, on a separate sheet, please insert a copy of the abstract which attached hereto.

REMARKS

The purpose of this preliminary amendment is insert a priority claim into the application and to provide a copy of the abstract on a separate sheet.

Favorable consideration leading to prompt allowance of the present application is respectfully requested.

Respectfully submitted,  
DANN, DORFMAN, HERRELL AND SKILLMAN  
A Professional Corporation

By   
Kathleen D. Rigaut, Ph.D., J.D.  
PTO Registration No. 43,047

Telephone: (215) 563-4100  
Facsimile: (215) 563-4044

**ABSTRACT**

A site-specific recombination system and methods of use thereof are disclosed for manipulating the genome of higher plants.

10/088634

20P/5  
WO 01/21768

PCT/US00/25930

Rec'd PCT/PTO 20 MAR 2002

Site-specific recombination system to manipulate the  
plastid genome of higher plants

5

This application claims priority under 35 U.S.C.  
10 §119(e) to US Provisional Applications 60/155,007 and  
60/211,139 filed September 21, 1999 and June 13, 2000  
respectively, the entire disclosure of each of the  
above-identified applications is incorporated by  
reference herein.

15

#### FIELD OF THE INVENTION

20 This invention relates to the fields of transgenic  
plants and molecular biology. More specifically, DNA  
constructs and methods of use thereof are provided which  
facilitate the excision of target DNA sequences from  
transplastomic plants.

#### BACKGROUND OF THE INVENTION

25 Several publications are referenced in this  
application by author name and year of publication in  
parentheses in order to more fully describe the state of  
the art to which this invention pertains. Full  
citations for these reference can be found at the end of  
30 the specification. The disclosure of each of these  
publications is incorporated by reference herein.

The plastid genetic system of higher plants is  
highly polyploid. For example, in a tobacco leaf there  
are as many as 100 chloroplasts, each carrying ~100  
35 identical genome copies, a total of 10,000 copies in a  
leaf cell. High-level protein expression, lack of  
pollen transmission and the feasibility to engineer  
polycistronic expression units make the plastid genome

an attractive alternative to nuclear engineering.

Plastid transformation vectors often contain a selective marker, most commonly a spectinomycin resistance (*aadA*) gene, flanked by plastid DNA sequences targeting insertion of the marker gene by homologous recombination into the plastid genome. Genes of commercial value but lacking a selectable phenotype are physically linked to the selective marker and the two genes are integrated together as a block of heterologous sequences. Plastid transformation is accomplished by biolistic DNA delivery or polyethylene glycol induced uptake of the transforming DNA followed by selection for the antibiotic resistance marker to ensure preferential propagation of plastids with transformed genome copies.

As the result, all the 10,000 wild-type plastid genome copies in a cell are replaced with transgenic copies during a gradual process (Maliga, 1993).

Incorporation of a selectable marker gene is essential to ensure preferential maintenance of the transformed plastid genome copies. However, once transformation is accomplished, maintenance of the marker gene is undesirable. One problem may be the metabolic burden imposed by the expression of the selectable marker gene. For example FLARE-S, the product of the marker gene with good prospects to transform cereal chloroplasts, accumulates up to 18% of the total soluble cellular protein (Khan and Maliga 1999). The second problem is the relatively high potential for horizontal transfer of plastid marker genes to microbes (Tepfer 1989; Dröge et al. 1998; Sylvanen 1999), as commonly used plastid maker gene constructs are efficiently expressed in *E. coli* (Carrer et al. 1993; Svab and Maliga 1993). Therefore, having plastid marker genes in commercial products is undesirable.

**SUMMARY OF THE INVENTION**

In accordance with the present invention, methods and systems are provided which facilitate the manipulation of the plastid genomes of higher plants.

The methods and systems of the invention may be employed to remove heterologous sequences from the plastid genome, such as selectable marker genes following successful isolation of transformed progeny.

Alternatively, they may be designed to remove endogenous genes involved in plant cell metabolism, growth, development and fertility.

In one embodiment of the invention, a site specific recombination method for removal of predetermined nucleic acid sequences from the plastid genome is provided. The method comprises providing a first nucleic acid construct, the construct comprising a promoter being operably linked to a nucleic acid encoding an optional plastid targeting transit sequence which is in turn operably linked to a nucleic acid encoding a protein having excision activity, the construct further comprising a first selectable marker encoding nucleic acid having plant specific 5' and 3' regulatory nucleic acid sequences. The method also entails the use of a second DNA construct, the second construct comprising an second selectable marker encoding nucleic acid and excision sites. The second construct optionally contains a gene of interest and further comprises flanking plastid targeting nucleic acid sequences which facilitate homologous recombination into said plastid genome. The second DNA construct is introduced into plant cell and the cells are cultured in the presence of a selection agent, thereby selecting for those plant cells expressing the proteins encoded by said second DNA construct. The first DNA construct is

then introduced into cells having the second construct in the presence of a selection agent and those plant cells expressing proteins encoded by said first construct are selected. If present, the excising activity acts on the excision sites, thereby excising said predetermined target sequence. Plants may then be regenerated from plant cells obtained by the foregoing method.

Proteins having excision activity suitable for the practice of the invention include, without limitation, CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase. Sequences corresponding to excision sites suitable for the practice of the invention, include, for example, LOX sequences, and *frt* sequences.

A variety of selection of agents may be selected. These include without limitation, kanamycin, gentamycin, spectinomycin, streptomycin and hygromycin, phosphinotricin, basta, glyphosate and bromoxynil.

In an alternative embodiment, a site specific recombination method for removal of predetermined nucleic acid sequences from the plastid genome is provided. The method comprising providing a first nucleic acid construct, said construct comprising a regulated promoter being operably linked to a nucleic acid encoding an optional plastid targeting transit sequence which is operably linked to a nucleic acid encoding a protein having excision activity, said construct optionally further comprising a first selectable marker encoding nucleic acid having plant specific 5' and 3' regulatory nucleic acid sequences. A second DNA construct is also provided, said second construct comprising an second selectable marker encoding nucleic acid and excision sites, said second construct further comprising flanking plastid targeting nucleic acid sequences which facilitate homologous

recombination into said plastid genome at a predetermined target sequence such that excision sites flank said predetermined target sequence following homologous recombination and introducing said second DNA construct into a plant cell. The plant cell so generated is then cultured in the presence of a selection agent, thereby selecting for those plant cells expressing the proteins encoded by said second DNA construct. A plant is then regenerated from cells containing the second construct and the first DNA construct is introduced into these cells in the presence of a selection agent and those plant cells expressing proteins encoded by said first construct are selected. The excising activity then acts on the excision sites, thereby excising said predetermined target sequence.

Regulatable promoters suitable for this embodiment of the invention include, without limitation, inducible promoters, tissue specific promoters, developmentally regulated promoters and chemically inducible promoters.

Candidate predetermined target sequences, may include for example genes associated with male sterility, *clpP*, ribosomal proteins, ribosomal operon sequences.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram depicting CRE-mediated excision and integration of DNA segments.

Figure 2 is a map of a plastid transformation vector pSAC48, with *codA* bracketed by direct *loxP* sites. Positions of plastid genes *rrn16*, *trnV*, *rps12/7* (Shinozaki et al. 1986), the *aadA* and *codA* transgenes and relevant restriction sites are marked.

Figure 3 is a map of an *Agrobacterium* binary vector

pPZP212 with a plastid-targeted *Ssu-tp-cre* gene. Marked are: *Agrobacterium* Left and Right Border fragments; the kanamycin resistance (*neo*) gene; P2' promoter; SSU transit peptide (*ssu-tp*); *cre* coding region; recognition sequences for restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Nhe*I and *Xba*I.

Figure 4 shows maps of the plastid genome >*codA*> deletion derivatives. Shown are the plastid targeting region of vector pSAC48; the map of same region of the wild-type plastid genome (Nt-wt); the map of the plastid genome with CRE-mediated deletion of *codA* via the *lox* sites; and the map of the plastid genome with deletion via Prrn sequences lacking *trnV*, *aadA* and *codA*.  
10 Positions of plastid genes *rrn16*, *trnV* and *rps12/7* (Shinozaki et al. 1986), *aadA* and *codA* transgenes, primers (O1-O4) and relevant restriction sites (AI, ApaI; EV, EcoRV) are marked.  
15

Figure 5 is a gel showing PCR amplification which confirms CRE-mediated deletion of *codA* from the plastid genome. Primers O1 and O2 (Fig. 3) amplified the 0.7-kb fragment of the deleted region. Same primers amplify the 2.0-kb *aadA-codA* fragment in tester lines Nt-pSAC48-21A and Nt-pSAC-16C (no transgenic Cre gene). No specific fragment was obtained in wild-type DNA sample and in Cre1-10 line. The lines obtained are listed in Table 1.  
20  
25

Figure 6 shows the results of DNA gel blot analysis wherein plastid genome structure was determined in the indicated plant samples. Total cellular DNA was isolated from the leaves of plants listed in Table 1 and digested with the ApaI and EcoRV restriction endonucleases. The probes were the wild-type ApaI-EcoRV plastid targeting

region and the *aadA* (*NcoI-XbaI* fragment) and *codA* (*NcoI-XbaI* fragment) coding regions. The hybridizing fragments are marked in Fig. 3.

5       Figure 7 are gels showing uniformity of plastid genome populations in the *Ssu-tp-cre* transformed plants. Total cellular DNA extracted from several leaves was probed with the *Apal-EcoRV* targeting region probe. Numbers identify leaves from which DNA was extracted.  
10      For example, seven different leaves were probed from the Cre1-3 plant. For details, see Brief Description of Fig. 6.

15      Figures 8A and 8B are gels of PCR analysis confirming CRE-mediated deletion of *codA* in seedlings obtained by pollination with *Ssu-tp-cre* activator lines. 5-day old seedlings were tested from the cross Nt-PSAC48-21A as maternal parent and Cre2-200 and Cre2-300 activator lines as pollen parents. Amplification products are also shown for controls Nt-pSAC48-21A selfed seedling (48 self), wild-type (wt), the parental plant (48P) and the Cre1-3 plant. Fig. 8A: The *codA* region was amplified with the O1/O2 primers: the size of *aadA-codA* fragment is 2.0 kb; the *codA* deletion fragment is 0.7 kb (Fig. 4). Fig. 8B: Testing for *cre* sequences by PCR amplification with the Cre1/Cre3 oligonucleotides.  
20  
25

30      Figure 9 is a diagram of the plastid transformation PSAC38 with the >*neo*< bracketed by inverted lox sites. Positions of plastid genes *rrn16*, *trnV* and *rps12/7* (Shinozaki et al., 1986), the *aadA* and *codA* transgenes and relevant restriction sites are marked.  
35

Figure 10 shows a map of the plastid genome

containing the >neo< inversion construct. Shown are the plastid targeting region of vector pSAC38; the map of the same region of the wild-type plastid genome (Nt-wt); map of the plastid genome with CRE-mediated inversion of neo via the lox sites. Positions of the plastid genes *rrn16*, *trnV* and *rps12/7* (Shinozaki et al., 1986) *aadA* and *neo* transgenes, primers (O1-O4) and relevant restriction sites (BamHI) are marked.

Figure 11 shows the results of DNA gel blot analysis for the determination of plastid genome structure of CRE-activated >neo< plants by DNA gel blot analysis. Total cellular DNA was digested with the BamHI restriction endonuclease. The probes was the wild-type *ApaI-EcoRV* plastid targeting region. The hybridizing fragments are marked in Fig. 10.

Figure 12 shows an exemplary monocistronic inversion vector. The gene of interest (*goi*) coding region is flanked by inverted lox sites (triangles). CRE activates *goi* expression by inversion, so that the coding strand is transcribed. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

Figure 13 shows an alternative dicistronic lox inversion vector. Note that the inverted lox sites flank the selective marker (*aadA*) and *goi*, and only one gene is expressed. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

Figure 14 shows a basic tobacco plastid lox deletion vector. The vector provides is a suitable backbone for vector construction and targets insertions into the *trnV-rps12/7* intergenic region.

Figure 15 shows a tobacco plastid *lox >aadA>* deletion vector. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

5

Figure 16 shows a tobacco constitutive *>aadA>goi* dicistronic deletion vector. *rrn16*, *trnV* and *rps12/7* are plastid genes and are described in (Shinozaki et al. 1986).

10

Figure 17 shows a tobacco constitutive *goi >aadA>* dicistronic deletion vector. Note that vectors shown in Fig. 16 and Fig. 17 differ in the relative order of marker gene and the gene of interest. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

15

Figure 18 shows a tobacco constitutive *goi >aadA>* dicistronic deletion vector, in which expression of *aadA* is dependent on translational coupling. Note that in this construct only one leader sequence is utilized. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986).

20

Figure 19 shows a tobacco inducible *lox* deletion vector. Expression of *goi* is dependent on *aadA* excision. *rrn16*, *trnV* and *rps12/7* are plastid genes (Shinozaki et al. 1986). Abbreviations: P, promoter; T, 3' untranslated region; L1 is 5' leader sequence.

25

Figure 20 shows a vector suitable for Cre-mediated deletion of *clpP* gene from the plastid genome. The region of engineered plastid genome shown is the sequence contained in the plastid transformation vector. The *clpP* Exons are dark boxes, the Introns are open

boxes. Map position of plastid genes *psbB*, *rps12* Exon I and *rpl20* is also shown.

#### **DETAILED DESCRIPTION OF THE INVENTION**

5       The following definitions are provided to aid in understanding the subject matter regarded as the invention.

10      Heteroplasmic refers to the presence of a mixed population of different plastid genomes within a single plastid or in a population of plastids contained in plant cells or tissues.

15      Homoplasmic refers to a pure population of plastid genomes, either within a plastid or within a population contained in plant cells and tissues.

20      Homoplasmic plastids, cells or tissues are genetically stable because they contain only one type of plastid genome. Hence, they remain homoplasmic even after the selection pressure has been removed, and selfed progeny are also homoplasmic. For purposes of the present invention, heteroplasmic populations of genomes that are functionally homoplasmic (i.e., contain only minor populations of wild-type DNA or transformed genomes with sequence variations) may be referred to herein as "functionally homoplasmic" or "substantially 25 homoplasmic." These types of cells or tissues can be readily purified to a homoplasmic state by continued selection.

Plastome refers to the genome of a plastid.

30      Transplastome refers to a transformed plastid genome.

Transformation of plastids refers to the stable integration of transforming DNA into the plastid genome that is transmitted to the seed progeny of plants containing the transformed plastids.

35      Selectable marker gene refers to a gene that upon

expression confers a phenotype by which successfully transformed plastids or cells or tissues carrying the transformed plastid can be identified.

Transforming DNA refers to homologous DNA, or heterologous DNA flanked by homologous DNA, which when introduced into plastids becomes part of the plastid genome by homologous recombination.

Operably linked refers to two different regions or two separate genes spliced together in a construct such that both regions will function to promote gene expression and/or protein translation.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule or its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e.,

in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

5 The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

10 The term "functional" as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

15 The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID No.: For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

20 A "replicon" is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

25 A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

30 An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than 5 three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either 10 RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either 15 single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide 20 probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid 25 sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact 30 complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer 35 sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity

with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the

desired template strand to functionally provide a template-primer complex for the synthesis of the extension product. Amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L-amino acid residue, provided the desired properties of the polypeptide are retained.

All amino-acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

The term "tag," "tag sequence" or "protein tag" refers to a chemical moiety, either a nucleotide, oligonucleotide, polynucleotide or an amino acid, peptide or protein or other chemical, that when added to another sequence, provides additional utility or confers useful properties, particularly in the detection or isolation, to that sequence. Thus, for example, a homopolymer nucleic acid sequence or a nucleic acid sequence complementary to a capture oligonucleotide may be added to a primer or probe sequence to facilitate the subsequent isolation of an extension product or hybridized product. In the case of protein tags, histidine residues (e.g., 4 to 8 consecutive histidine residues) may be added to either the amino- or carboxy-terminus of a protein to facilitate protein isolation by chelating metal chromatography. Alternatively, amino acid sequences, peptides, proteins or fusion partners representing epitopes or binding determinants reactive with specific antibody molecules or other molecules (e.g., flag epitope, c-myc epitope, transmembrane epitope of the influenza A virus hemagglutinin protein, protein A, cellulose binding domain, calmodulin binding protein, maltose binding protein, chitin binding domain, glutathione S-transferase, and the like) may be added to proteins to

facilitate protein isolation by procedures such as affinity or immunoaffinity chromatography. Chemical tag moieties include such molecules as biotin, which may be added to either nucleic acids or proteins and facilitates isolation or detection by interaction with avidin reagents, and the like. Numerous other tag moieties are known to, and can be envisioned by, the trained artisan, and are contemplated to be within the scope of this definition.

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radioimmunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion, biolistic bombardment and the like.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

5 A "cell line" is a clone of a primary cell or cell population that is capable of stable growth *in vitro* for many generations.

#### **CRE-MEDIATED SITE SPECIFIC RECOMBINATION**

The plastid genome of higher plants is present in 10 100-10,000 copies per cell. Incorporation of a selectable marker gene is essential to ensure preferential maintenance of the transformed plastid genome copies carrying useful genes with no selectable phenotype. However, once transformation is accomplished, 15 maintenance of the marker gene is undesirable. In accordance with the present invention, a bacteriophage P1CRE-loxP site-specific recombination system is provided which is suitable for efficient elimination of marker genes from the plastid genome. The system 20 exemplified herein has two components: a plastid tester strain carrying a cytosine deaminase (*codA*) transgene flanked by *lox* sites conferring sensitivity to 5-fluorocytosine and a nuclear CRE line carrying a nuclear-encoded, plastid-targeted CRE. Both the plastid 25 tester (no CRE activity) and the nuclear CRE line (no *lox* sequence) were genetically stable. However, *codA* was eliminated at a very fast rate when the plastid-targeted CRE was introduced into the plastid tester strain by transformation or crossing. The gene for the nuclear- 30 encoded CRE was subsequently separated from the transformed plastids by segregation in the seed progeny. Excision of *codA* by CRE was often accompanied by deletion of a plastid genome segment flanked by short 35 directly repeated sequences. Removal of the antibiotic resistance marker from the transplastomic plants

eliminates the metabolic burden imposed by the expression of the selectable marker gene and should also improve public acceptance of the transgenic crops.

Additional applications of the CRE-lox site-specific recombination system are activation of plastid gene expression by deletion or inversion of plastid genome sequences and induction of controlled cell death by deleting vital genes in the male reproductive tissue.

Although the use the CRE recombinase is exemplified herein, other prokaryotic and eukaryotic site-specific recombinases would be equally suitable for the elimination of the marker genes.

Recently, several prokaryotic and lower eukaryotic site-specific recombination systems have been shown to operate successfully in higher eukaryotes. In plant and animal cells functional site-specific recombination systems from bacteriophages P1 (*Cre-lox*) Mu (*Gin-gix*), and from the inversion plasmids of *Saccharomyces cerevisiae* (*FLP-frt*) (Morris et al. 1991; O'Gorman et al. 1991; Lichtenstein and Barrena 1993; Lyznik et al. 1993; Lyznik et al. 1995; Lyznik et al. 1996) and *Zygosaccharomyces rouxii* (R-RS). In each of these systems, no additional factor aside from the recombinase and target sequences is required for recombination.

Reviewed in van Haaren and Ow, 1993. The CRE-*loxP* site-specific recombination system of bacteriophage P1 has been studied extensively *in vitro* and in *E. coli* (Craig 1988; Adams et al. 1992). Expression of the CRE protein (38.5 kDa) is sufficient to cause recombination between 34 bp *loxP* sites that consist of 13 bp inverted repeats separated by 8 bp asymmetric spacer sequence. If there are two *loxP* sites within a DNA segment, the result of the recombination reaction depends on the relative position of the recombination sites. If the recombination sites form a direct repeat, that if they

are in the same orientation, recombination results in deletion of the intervening DNA. If the recombination sites are in an inverted orientation, CRE-mediated recombination results in an inversion of the intervening 5 DNA. The products of these reactions are shown in Fig. 1. The CRE site-specific recombination system has been employed for the elimination of nuclear genes in a number of eukaryotic systems, including higher plants (Dale and Ow 1991; Russell et al. 1992; Srivastava et 10 al. 1999).

Before the present invention, the efficiency of CRE-mediated elimination of targeted plastid genes was unknown. To explore this system for this purpose, CRE-mediated elimination of the *codA* gene encoding cytosine 15 deaminase (CD; EC 3.5.4.1) was assessed. Cytosine deaminase converts 5-fluorocytosine (5FC) into 5-fluorouracil (5FU), the precursor of 5-fluoro-dUMP. 5FC is lethal for CD-expressing cells due to irreversible inhibition of thymidylate synthase by 5-fluoro-dUMP 20 (Beck et al. 1972). Cytosine deaminase is absent in plants. Expression of the bacterial *codA* in plastids renders cells sensitive to 5FC, while cells deficient in transgene expression are resistant (Serino and Maliga 1997). Thus, 5FC resistance could be used for positive 25 identification of cells with CRE-induced *codA* deletion, even if such deletion events were relatively rare. The test system of the present invention incorporates a *codA* gene in the tobacco plastid genome between two 30 directly oriented *lox* sites (>*codA*>). The transplastome was stable in the absence of CRE activity. However, highly efficient elimination of >*codA*> was triggered by introduction of a nuclear-encoded plastid-targeted CRE.

**CRE-MEDIATED DELETION OF THE SELECTABLE PLASTID MARKER**

Cre-mediated deletion of the selective plastid marker in the plastids of tobacco somatic cell is described in Example I. The selectable marker flanked by the lox sites is exemplified here by codA. However, it could be any other selectable and non-selectable marker gene, or any DNA sequence independent of information content flanked by lox sites in the palstid genome. Components of the test stystem are tobacco plants carrying a codA coding region flanked by lox sites (>codA>). A second component of the test system is a nuclear gene encoding a plastid targeted CRE-site specific recombinase. Deletion of a plastid encoded >codA> is achieved by introducing nuclear Cre into the nucleus of somatic (leaf) tobacco cells by Agrobacterium-mediated transformation. Alternatively, the nuclear encoded Cre gene may be introduced by fertilization with pollen of an appropriate activator-of-deletion strain. The nuclear Cre gene is subsequently removed by segregation in the seed progeny.

**MATERIALS AND METHODS FOR THE PRACTICE OF EXAMPLE 1**

The following materials and methods are provided to facilitate the practice of Example 1.

**Plastid codA with direct lox sites.**

The codA gene is contained in a SacI-HindHIII fragment.

The gene map is shown in Fig. 2. PrrnloxD (Seq. ID No. 4) is a plastid rRNA operon (rrn16) promoter derivative. It is contained in a SacI-EcoRI fragment obtained by PCR using oligonucleotides 5'-

GGGGAGCTCGCTCCCCGCCGTCGTTCAATG-3' and 5'-

GGGAATTCTATACTTCGTATAGCATACATTATACGAAGTTAT

GCTCCCAGAAATATAGCCA-3' as primers and plasmid pZS176 (progenitor of plasmid pZS197; Svab and Maliga 1993) as a template. The promoter fragment PrrnloxD contains a lox site at the 3' end adjacent to the EcoRI site. The 5 EcoRI-NcoI fragment contains the ribosome binding site from plasmid pZS176. The fragment was obtained by annealing the complementary oligonucleotides 5'-AATTGGAAGCGCTTGGATAACAGTTGTAGGGAGGGATC-3' and 5'-CATGGATCCCTCCCTACAACGTATCCAAGCGCTTCG-3'. The codA 10 coding region is contained in an NcoI-XbaI fragment (Serino and Maliga 1997). The TrbcLloxD (Seq. ID No. 5) is the rbcL 3'-untranslated region contained in an XbaI-HindIII fragment obtained by PCR using oligonucleotides 5'-GGTCTAGATAACTTCGTATAATGTATGCTATA 15 CGAAGTTATAGACATTAGCAGATAAATT-3' and 5'-GGGGGTACCAAGCTTGCTAGATTTGTATTCAAATCTTG-3' and plasmid pMSK48 (Khan and Maliga 1999) as template. TrbcLloxD contains a lox site adjacent to the XbaI site in direct orientation relative to the lox site in the codA 5'UTR. 20 The chimeric PrrnloxD:codA:TrbcLloxD gene was introduced into the tobacco plastid transformation vector pPRV111B (Zoubenko et al. 1994) as a SacI-HindIII fragment to obtain plasmid pSAC48.

25 **Plastid-targeted nuclear cre linked to a nuclear kanamycin resistance gene.** Two plastid targeted nuclear cre genes were tested. The cre gene in Agrobacterium binary vector pKO27 and pKO28 encode the CRE recombinase at its N terminus translationally fused with the pea Rubisco small subunit (SSU) chloroplast transit peptide (Timko et al. 1985) and twenty two and five amino acids of the mature Rubisco small subunit, respectively. Both cre genes are contained in an EcoRI-HindIII fragment. 30 The schematic map of the genes is shown in Fig. 3. The

P2' Agrobacterium promoter (Velten et al. 1984) (Sequence ID. No.9) is contained in an EcoRI-NcoI fragment. The P2' promoter fragment was obtained by PCR using oligonucleotides 5'-  
5 ccgaattcCATTTCACGTGTGGAAGATATG-3' and 5'-  
ccccatqqttagqatcctatCGATTGGTGTATCGAGATTGG-3' as primers and plasmid pHCl (Carrer et al. 1990) as template. PCR amplification introduced an EcoRI site at the 5' end and ClaI, BamHI and a NcoI sites at the 3' end. A T  
10 introduced between the ClaI and the BamHI sites eliminates an ATG and introduces an in-frame stop codon (Sriraman 2000). The Rubisco SSU transit peptides are included in BamHI-NcoI fragments. The pKO27 fragment (Pea SSU-TP22; Sequence ID No. 7) was obtained by using oligonucleotides 5'-CCGGATCCAATTCAACCACAAGAACTAAC-3' and 5'-GGGGCTAGCCATGGCAGGCCACACCTGCATGCAC-3' as primers and plasmid pSSUpGEM4 as the template (Timko et al. 1985). The pKO28 fragment (Pea SSU-TP5; Sequence ID No. 6) was obtained by using oligonucleotides 5'-  
15 CCGGATCCAATTCAACCACAAGAACTAAC-3' and 5'-  
GGGGCTAGCCATGGTCAATGGGTTCAAATAGG-3' as primers and plasmid pSSUpGEM4 as the template (Timko et al. 1985). A pea SSU-TP with 23 amino acids of the mature polypeptide is shown in Sequence ID No. 8. The cre coding region  
20 included in a NcoI-XbaI fragment (Sequence ID No. 3) was obtained by PCR amplification using the Cre1 5'-  
GGGGAGCTCCATGGCTAGCTCCAATTTACT  
25 GACCGTACAC-3' and Cre2 5'-GGGTCTAGACTAATGCCATC  
CTCGAGCAGGCGCACCATTC-3' oligonucleotides as primers and DNA isolated from *Escherichia coli* strain BNN132 (ATCC  
30 number 47059) as template. The presence of cre gene in plant nuclear DNA was confirmed by PCR amplification with the Cre 1 and Cre3 oligonucleotides. The sequence of Cre3 oligonucleotide is 5'-TCAATCGATGAGTTGCTTC-3'.  
35 The *Agrobacterium nos* terminator (Tnos) is included in a

XbaI-HindIII fragment (Svab et al. 1990). The plastid targeted nuclear *cre* genes were introduced as EcoRI-HindIII fragments into the pPZP212 *Agrobacterium* binary vectors (Hajdukiewicz et al. 1994) to obtain plasmids pKO27 and pKO28 with twenty two and five amino acids of the mature Rubisco SSU. A schematic map of the *Agrobacterium* vectors is shown in Fig. 3.

**Transgenic plants.** Plastid transformation using the biolistic protocol, selection of transplastomic tobacco clones (RMOP medium, 500 mg/L spectinomycin dihydrochloride) and characterization of the transplastomic clones by DNA gel blot analysis was described (Svab and Maliga 1993). Transformation with *Agrobacterium* vectors pKO28 or pKO27 and regeneration of transformed tobacco plants has also been reported (Hajdukiewicz et al. 1994). Briefly, nuclear gene transformants were selected by kanamycin resistance on RMOP shoot regeneration medium containing 100 mg/L kanamycin and 500 mg/L carbenicillin. Kanamycin resistance of the shoots was confirmed by rooting on plant maintenance (RM) medium containing 100 mg/L kanamycin. Testing of 5FC cytotoxicity was carried out on RMPO medium according to published procedures (Serino and Maliga 1997).

**Transplastomic tobacco plants with a *codA* gene flanked by direct lox sites.**

Plastid transformation vector pSAC48 carries a *codA* gene in which two lox sites flank the coding region in a direct orientation. If the *codA* coding region is deleted via the lox sites, a lox site flanked by the promoter (Prrn) and terminator (TrbcL) are left behind. The selective marker in pSAC48, a pPRV111B vector

derivative, is a spectinomycin resistance (*aadA*) gene (Fig. 2). Transformation with plasmid pSCAC48 yielded a number of independently transformed transplastomic lines, of which four were purified to the homoplastomic state: Nt-pSAC48-21A, Nt-pSAC48-16C, Nt-pSAC48-16CS and Nt-pSAC48-9A. These lines are considered identical other than they have been generated independently. A uniform population of transformed plastid genomes in the transplastomic plants was verified by DNA gel blot analysis (see below).

**Nuclear-encoded plastid-targeted Cre genes.**

To activate deletion of the plastid >*codA*> gene we introduced an engineered *cre* gene into the nucleus of the transplastomic lines encoding a plastid-targeted CRE. Targeting of nuclear-encoded plastid proteins is by an N-terminal transit peptide (TP) cleaved off during import from the cytoplasm into plastids (Soll and Tien, 1998). To ensure plastid targeting of the CRE recombinase, it was translationally fused with the Rubisco small subunit (SSU) transit peptide (Timko et al. 1985). Therefore, the product of the protein fusion is SSU-TP-CRE. Efficiency of import of chimeric proteins depends on the size of mature protein N-terminus incorporated in the construct (Wasemann et al. 1986; Lubben et al. 1989). Two chimeric *cre* genes (*Ssu-tp-cre*) were prepared, one with 5 (vector pKO28) and one with 22 (plasmid pKO27) amino acids of the mature SSU N-terminus, encoding SSU-TP5-CRE and SSU-TP22-CRE, respectively. These genes are also referred to as *Cre1* and *Cre2*, respectively (Table 1). The *cre* genes were expressed in the P2' promoter and Tnos terminator cassettes in the *Agrobacterium* pPZP212 binary vector which carries kanamycin resistance (*neo*) as a selectable marker (Fig. 3).

Tobacco plant transformed with *Ssu-tp5-cre* (pKO37) and *Ssu-tp22-cre* (pKO36) were also obtained. In these plants the nuclear *cre* is expressed from the cauliflower mosaic virus 35S promoter (Seq. ID No. 10; Timmermans et al. 1990).

Line	Plastid genotype <sup>a</sup>	Nuclear marker
Wild-type	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	
Nt-pSAC48-21A	<i>trnV+</i> <i>aadA+</i> <i>codA+</i>	
Nt-pSAC48-16C		
Cre1-1	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre1-2	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre1-3	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre1-4	<i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre1-10	<i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre2-1	<i>trnV+</i> <i>aadA+</i> <i>codA-</i>	<i>neo</i>
Cre2-2	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV+</i> <i>aadA*+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre2-3	<i>trnV+</i> <i>aadA+codA+</i> <i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV+</i> <i>aadA*+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre2-4	<i>trnV+</i> <i>aadA+</i> <i>codA-</i>	<i>neo</i>
Cre2-5	<i>trnV+</i> <i>aadA+</i> <i>codA-</i>	<i>neo</i>
Cre2-10	<i>trnV+</i> <i>aadA+</i> <i>codA-</i> <i>trnV-</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre1-100	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre2-100	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre2-200	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>
Cre2-300	<i>trnV+</i> <i>aadA-</i> <i>codA-</i>	<i>neo</i>

30

<sup>a</sup>Presence or absence of plastid gene is indicated by + or -. Since the plastid *trnV* gene is deleted in some of the lines, the wild-type plastid genotype is *trnV+* *aadA-* *codA-*.

35

**Deletion of codA from the plastid genome in somatic**

cells.

To test the efficiency of CRE-mediated deletion in somatic cells, the *Ssu-tp-cre* genes were introduced into the nucleus of the transplastomic >*codA*> lines by

5 cocultivation of *Agrobacterium* and tobacco leaf disks.

Plants representing 11 individual *Ssu-tp-cre* insertion events have been characterized. Five lines (Cre1-derivatives) were obtained by transformation with *Ssu-tp5-cre* gene (vector pKO28) and six lines (Cre2-derivatives) were obtained by transformation with the 10 *Ssu-tp22-cre* (vector pKO27) (Table 1).

Deletion of *codA* was first tested in a DNA sample taken from one leaf of eleven kanamycin resistant shoots representing an individual integration event of the 15 nuclear *Cre* gene. Subsequently, 4 to 7 additional leaves were sampled from six shoots to confirm that the result of the analysis is typical for the plant.

The initial DNA samples were first screened for the loss of >*codA*> by PCR using the O1/O2 primer pair

20 complementary to sequences in the *aadA* coding region N terminus and the *codA* promoter (Fig. 4A). Amplification with these primers yields a ~0.7-kb fragment if >*codA*> is deleted and a ~2.0-kb fragment if the >*codA*> gene is still present. Ethidium bromide stained gels of PCR 25 products in Fig. 5 indicate complete loss of >*codA*> in each of the samples. A perfect, reconstituted *lox* site between Prrn and TrbcL was confirmed in eight clones by PCR amplification of the region with primers O1/O4 from the same DNA samples and direct sequencing of the 30 amplification product with primer O2 (not shown). In two clones (Cre1-4, Cre1-10) a fragment is missing due to deletion of *aadA* alongside with *codA* (see below). Plastid genome structure in the initial DNA sample was determined by gel blot analysis of *ApaI-EcoRV* digested

total cellular DNA. The probes were the plastid targeting region and the *aadA* and *codA* coding regions. The DNA gel blots are shown in Fig. 6. The maps of the parental genomes and deletion derivatives that help to interpret these genomes are shown in Fig. 4. In the 5 plastid tester strains expressing no CRE (Nt-pSAC48-21A, Nt-pSAC48-16C) all three probes hybridized to the same 4.9-kb DNA fragment consistent with both *codA* and *aadA* being present in all the plastid genome copies. In the 10 SSU-TP-CRE expressing plants no 4.9-kb fragment was detectable indicating the dramatic speed by which the >*codA*> gene was eliminated from the plastid genome. CRE-mediated deletion of >*codA*> via the *lox* sites yielded the 3.6-kb fragment detected in nine of the eleven 15 clones. The 3.6-kb fragment was the only product detected in four clones, and was present in a heteroplasmic population in five clones. Unanticipated was formation of a 1.4-kb *ApaI-EcoRV* fragment in five clones. DNA gel blot analysis confirmed that this 20 fragment lacks both *codA* and *aadA*, and is smaller than the wild type *ApaI-EcoRV* fragment (1.9-kb). Direct sequencing of PCR products in this region confirmed deletion of *codA*, *aadA* and *trnV* by homologous recombination via the duplicated *Prrn* promoter regions. 25 One of the *Prrn* promoters is driving *codA*, the other is upstream of the rRNA operon at its native location. Deletion of *trnV* is the reason why the *ApaI-EcoRV* fragment derived from this region (1.4-kb) is smaller than the wild-type fragment (1.9-kb). 30 The initial DNA samples were taken from one leaf of a plant obtained by rooting the shoot obtained after transformation with the *Ssu-tp-cre* genes. To confirm that the DNA samples extracted from the leaf were typical for the plant, we have sampled several more

leaves from the same plants (Fig. 7). In four clones 5 *codA* was excised by CRE via the *lox* sites, and the shoots were homoplasmonic for the deleted genome. Two of these, Cre1-3 and Cre2-4 were further characterized by testing seven and four additional leaves of the same plants, respectively. DNA gel blot analysis of these samples confirmed a uniform deletion of >*codA*> from all genome copies. These plants are the desired final products carrying the desired plastid transgenes and lacking the undesirable selective marker. These plants 10 and their progeny can be used directly for the production of recombinant proteins as they are free from the selectable marker gene. Furthermore, these plants are a source of engineered chloroplasts for introduction 15 into breeding lines by sexual crossing. The seed progeny of the plants is segregating for the *Ssu-tp-cre* activator gene. Plants with the desired chloroplasts but lacking the activator gene can be identified by PCR testing for *cre* sequences. Alternatively, individuals 20 lacking *cre* can be identified in the seed progeny by sensitivity to kanamycin, since the *Ssu-tp-cre* genes in the pK027 and pK028 Agrobacterium vectors are physically linked to kanamycin resistance (*neo* gene; Fig. 3). In two clones, Cre1-4 and Cre1-10, deletion of *trnV* 25 (encoding tRNA-Val<sup>GAC</sup>), *aadA* and *codA* occurred by homologous recombination via the duplicated Prrn promoter region. The Cre1-10 plant is homoplasmonic for the deletion based on probing seven additional leaves (Fig. 7). Apparently, the one remaining *trnV* gene 30 encoding tRNA-Val<sup>UAC</sup> is sufficient for the translation of all valine codons, or there is import of tRNA-Val<sup>GAC</sup> from the cytoplasm. In the Cre1-4 clone some of the leaves (two out of four) contained residual genome copies with *trnV* and *aadA*.

35 In five clones the initial DNA samples contained

more than one type of plastid genome copies. Mixed populations of plastid genome populations were confirmed in all parts of the plants by testing additional leaves (Fig. 7). Genetically stable *codA* deletion lines can be obtained from these heteroplasmonic plants by testing plants regenerated from single somatic cells or individual seedlings in a segregating seed progeny.

**10 Deletion of *codA* from the plastid genome in the seed progeny.**

CRE-mediate deletion of the negative plastid marker *codA* in somatic cells was described in the previous section. Deletion of the plastid marker gene in the somatic cells of the transplastomic plants, without going through a sexual cycle, is highly desirable to accelerate the production of marker-free transplastomic plants.

However, this approach is feasible only if there is a system for tissue culture and plant regeneration from somatic cells. Such system is unavailable for the economically important cereal crops rice and maize. As an alternative to transformation of somatic cells, we developed CRE activator lines carrying a nuclear-encoded plastid-targeted Cre to be used as the source of Cre gene when used as a pollen parent. The tobacco CRE activator lines were obtained by transforming the nucleus of wild-type plants with SSU-TP-CRE constructs. Lines in which the Cre is linked to a nuclear kanamycin resistance gene in a wild-type cytoplasm are Cre1-100, Cre2-100, Cre2-200 and Cre2-300 (Table 1).

To activate deletion of >*codA*> in the seed progeny, tester plants Nt-pSAC48-21A and Nt-pSAC48-16C were emasculated to prevent self fertilization, and fertilized with pollen from the Cre2-200 and Cre2-300 activator lines. The activator lines are primary transgenic plants ( $T_0$ ) segregating for the *Ssu-tp-cre*

gene. Therefore, a proportion of the seed progeny derived from the cross will have the activator genes while others will not. If the *codA* gene is present, the O1/O2 primer pair marked in Fig. 4 amplifies a 2.0-kb fragment. If the *codA* gene is absent, the same primers will amplify a 0.7-kb fragment. PCR analysis shown in Fig. 8 confirmed CRE-mediated deletion of >*codA*> in seedlings. The Cre1-100, Cre2-100 and Cre2-300 activator lines are apparently expressing CRE efficiently, indicated by the presence of only of the 0.7-kb fragment in seedlings carrying the nuclear *cre* gene. In seedlings with no *cre* sequence the same primers amplified the 2.0-kb *codA*-containing fragment. Interestingly, *cre*+ seedlings from the cross with Cre2-200 contained a mixed population of *codA* containing (2.0-kb) and *codA*-deleted (0.7-kb) fragments indicating less efficient CRE-induced deletion of >*codA*>. Thus, expression level and tissue specificity of the two nuclear *Ssu-tp22-cre* genes are characteristic for the individual transformation events. CRE activity of Cre1-100, Cre2-100 and Cre2-300 activator lines is more suitable for rapid elimination of >*codA*> in a cross than the Cre2-200 line. It is undesirable to maintain the *Ssu-tp-cre* activator genes in the production lines. However, these are encoded in the nucleus, and can be separated from the transgenic chloroplasts in the next seed progeny. Linkage of *Ssu-tp-cre* to the nuclear kanamycin resistance gene facilitates identification of seedlings lacking *cre* in a segregating seed population.

30

**CRE site-specific recombinase for deletion of plastid DNA sequences.** Biolistic transformation of tobacco leaves always yields shoots containing a mixed population of plastid genome copies. A mixed population

of plastid genome copies is determined by DNA gel blot analysis (Carrer et al. 1993; Svab and Maliga 1993; Carrer and Maliga 1995) and can be visualized in UV light when expressing the green fluorescence protein in 5 plastids (Khan and Maliga 1999). Homoplasmic, genetically stable plants are obtained during a second cycle of plant regeneration from the leaves of the regenerated plants or in the seed progeny. The cells of the >*codA*> tester strains carry a uniform population of 10 plastid genome copies. Thus, the *Ssu-tp-cre* is introduced into the nuclear genome of a cell that is homoplasmic for >*codA*>. It was expected that the regenerated shoots would contain a mixed population of 15 plastid genome copies. Instead, all plastid genome copies lack >*codA*>, an evidence for the enormous selection pressure by CRE activity against plastid genome copies that carry two *lox* sites. It is important that deletion of >*codA*> occurs in the absence of selection against >*codA*> by exposure to 5- 20 fluorocytosine. Virtually complete elimination of >*codA*> may also be obtained when CRE activity is introduced by crossing, using pollen of an appropriate deletion activator strain. Deletion of the selectable marker in somatic cells is the preferred choice over elimination 25 of the marker in the seed progeny. The most important advantage is time saving. Introduction of *Ssu-tp-cre* into the nucleus of somatic cells requires only three to six weeks; *Ssu-tp-cre* segregates out in the first seed progeny. In contrast, introduction and elimination of 30 *Ssu-tp-cre* takes one additional seed progeny, about three months.

Interestingly, genome copies with one *lox* site or no *lox* site (wild-type) are stable in CRE-expressing cells. Instability of genomes with two *lox* sites may be due to formation of linear ends during the excision 35

process. The linear ends may then re-circularize by homologous recombination via the Prrn promoter sequences yielding the *trnV-aadA-codA* deletion derivatives.

5       **CRE engineering.** Although CRE is a prokaryotic protein, it naturally carries a nuclear localization signal (NLS) that targeted a CRE-GFP fusion protein to the nucleus in mammalian cells. The NLS sequences overlap the DNA binding regions and the integrity of this region is  
10      important for DNA recombinase activity (Le et al. 1999). We targeted the newly-synthesized TP-CRE protein to plastids using a plastid-targeting transit peptide (TP). The TP is localized at the N terminus of plastid proteins and is cleaved off during import from the  
15      cytoplasm into plastids (Soll and Tien, 1998). Therefore, we translationally fused a plastid transit peptide with CRE to direct its import from the cytoplasm to plastids. Translational fusion yielded a protein with an N-terminal plastid targeting signal and an internal  
20      nuclear localization signal. Efficient CRE-mediated deletion of plastid-encoded *codA* genes indicates targeting of SSU-TP-CRE to plastids. When two potential targeting sequences are present, in general one of them out-competes the other (Small et al. 1998). N-terminal  
25      organelle targeting sequences normally dominate the second internal localization signal. For example, the 70-kDa heat shock protein of watermelon cotyledons that carry N-terminal plastidal and internal glyoxysomal targeting sequences are exclusively targeted to  
30      plastids. Proteins are localized to glyoxysomes only in the absence of the plastidal presequence (Wimmer et al. 1997). The tRNA modification enzymes contain information for both mitochondrial (N-terminal extension) and nuclear targeting. The enzyme with the N-terminal  
35      extension is targeted to mitochondria and only the short

form lacking the N-terminal extension is targeted to the nucleus (Small et al. 1998). It was fortunate, that the Rubisco SSU N-terminal transit peptide dominated the CRE nuclear localization signals and the TP-CRE fusion protein was directed to plastids (chloroplasts).

A second property that is important for the present invention is maintenance of recombinase activity when CRE is fused with proteins or peptides at its N and C termini. N-terminal fusion of CRE with the *E. coli* maltose binding protein did not interfere with recombinase function (Kolb and Siddell 1996). CRE was also shown to accept a C-terminal fusion with GFP (Le et al. 1999) as well as an 11-amino-acid epitope to the herpes simplex virus (HSV) glycoprotein D coat protein.

The epitope tag facilitates detection of CRE expression *in vitro* and *in vivo* using immunofluorescent labeling with a commercially available antibody (Stricklett et al. 1998). Apparently, the five and 22 amino acids that are left behind after processing of the SSU-TP5-CRE and SU-TP22-CRE proteins did not interfere with CRE function.

**Dominant negative selection markers for positive identification of deletion derivatives.** A practical application of the present invention is the removal of selectable marker genes from the transformed plastid genome. In tobacco, the excision process mediated by the CRE constructs described herein is so efficient that the >codA> deletion derivatives can be identified in the absence of 5FC selection. However, in other crops CRE-mediated excision of marker genes may be less efficient. In these species, the positive selective marker (aadA) may be fused with a dominant negative selective marker using linker peptides as described in the literature (Khan and Maliga 1999) or the positive and negative

marker genes may be combined in a dicistronic operon (Staub and Maliga 1995). Dominant negative selection markers allow normally non-toxic compounds to be used as toxic agents, so that cells which express these markers are non-viable in the presence of the compound, while cells that don't carry them are unaffected.

For example, cytosine deaminase is absent in plants. Expression of *codA*, encoding cytosine deaminase (CD; EC 3.5.4.1), in plastids renders tissue culture cells and seedlings sensitive to 5FC, facilitating direct identification of clones lacking this negative selective marker (Serino and Maliga 1997). Cytosine deaminase converts 5-fluorocytosine (5FC) into 5-fluorouracil (5FU), the precursor of 5-fluoro-dUMP. 5FC is lethal for CD-expressing cells due to irreversible inhibition of thymidylate synthase by 5-fluoro-dUMP (Beck et al. 1972). We have found that seedlings and plant tissues expressing >*codA*> were sensitive to 5FC. Seedlings lacking *codA* could be readily identified by 5FC resistance. Thus, the constructs described here are suitable to express cytosine deaminase at sufficiently high levels to be useful to implement a negative selection scheme.

Alternative negative selective markers can be obtained by adaptation of substrate-dependent negative selection schemes described for nuclear genes. Such negative selection schemes are based on resistance to indole, napthyl, or napthalene acetamide (Depicker et al. 1988; Karlin-Neumann et al. 1991; Sundaresan et al. 1995), chlorate (Nussaume et al. 1991), kanamycin (Xiang and Guerra 1993) and 5-fluorocytosine (5FC) (Perera et al. 1993; Stougaard 1993).

#### EXAMPLE 2

#### Cre-MEDIATED INVERSION OF PLASTID DNA SEQUENCES

If the lox sites in bacteria are in an inverted orientation, CRE-mediated recombination results in an inversion of the intervening DNA. We have tested, whether the CRE-mediated inversion reaction also occurs in plastids of higher plants containing DNA sequences flanked by inverted lox sites. This was assessed using a kanamycin-resistance (>neo<) coding region in an inverted orientation relative to the promoter (Fig. 9). In this construct the non-coding strand of neo is transcribed and the plants are kanamycin sensitive. The >neo< coding region is flanked by inverted lox sites. CRE-mediated inversion of the sequences reverses neo orientation resulting in the transcription of the sense strand and expression of kanamycin resistance. Inversion of the plastid-encoded >neo< coding region may be achieved by multiple approaches. One approach is to introduce a nuclear Cre into the nucleus of somatic tobacco cells, e.g., leaf, by *Agrobacterium*-mediated transformation. A second approach is introduction of the nuclear-encoded Cre gene by fertilization with pollen of an appropriate activator-of-inversion strain. Additional approaches are to provide CRE-activity via the incorporation of chemically inducible promoter into the construct, or to transiently express CRE from a nuclear or chloroplast construct.

**MATERIALS AND METHODS FOR THE PRACTICE OF EXAMPLE 2**

**Plastid neo gene with inverted lox sites.** The neo gene is contained in a *SacI-HindIII* fragment. The gene map is shown in Fig. 8. PrrnloxI (Seq. ID No. 1) is a plastid rRNA operon (*rrnl6*) promoter derivative. It is contained in a *SacI-XbaI* fragment obtained by PCR using oligonucleotides 5'-ggggagctcGCTCCCCCGCCGTCGTTCAATG-3' and 5'-ggtctagaataacttcgtatagcatacattatacgaagtatGCTCCC 35 AGAAATATAGCCA-3' as primers and plasmid pZS176

(progenitor of plasmid pZS197; Svab and Maliga 1993) as a template. The promoter fragment PrrnloxI contains a lox site at the 3' end adjacent to the XbaI site. The neo coding region is contained in an NcoI-XbaI fragment derived from plasmid pHC62. The neo sequence in plasmid pHC62 is identical with the neo sequence shown in Fig. 28B, US Patent 5,877,402. The EcoRI-NcoI fragment contains the ribosome binding site from plasmid pZS176. The fragment was obtained by annealing the complementary oligonucleotides 5'-  
10 AATTCGAAGCGCTTGGATAACAGTTGTAGGGAGGGATC-3' and 5'-  
CATGGATCCCTCCCTACAACGTATCCAAGCGCTTCG-3'. The TrbcLloxI  
(Seq. ID No. 2) is the rbcL 3'-untranslated region  
15 contained in an EcoRI-HindIII fragment obtained by PCR  
using oligonucleotides 5'-gggaattcataacttcgtatagcatacat  
tatacgaagttatAGACATTAGCAGATAAATT-3' and 5'-  
20 gggggtaccaaqcttgCTAGATTTGTATTCAAATCTTG-3' and plasmid  
pMSK48 (Khan and Maliga 1999) as template. TrbcLloxI  
contains a lox site adjacent to the EcoRI site in an  
inverted orientation relative to the lox site in  
25 PrrnloxI. The chimeric PrrnloxI:neo:TrbcLloxI gene was  
introduced into the tobacco plastid transformation  
vector pPRV111B (Zoubenko et al. 1994) as a SacI-HindIII  
fragment to obtain plasmid pSAC38.

25 **Plastid-targeted nuclear cre linked to a nuclear gentamycin resistance (aacC1) gene.** The plastid targeted nuclear cre genes were introduced as EcoRI-HindIII fragments into the pPZP222 Agrobacterium binary vectors which carry a plant-selectable gentamycin resistance gene (Hajdukiewicz et al. 1994) to obtain plasmids pKO30 and pKO31 with twenty two and five amino acids of the mature Rubisco SSU. The map of the Agrobacterium vectors is identical with the one shown in Fig. 3. other than

they carry a gentamycin resistance gene.

5           **Transplastomic tobacco plants with a *neo* gene flanked by inverted lox sites.**

Plastid transformation vector pSAC38 with the inverted >*neo*< gene is shown in Fig. 9. The inverted >*neo*< gene was introduced into plastids by selection for spectinomycin resistance (*aadA*) encoded in the vector.

10          Two independently transformed lines were purified to the homoplastomic state: Nt-pSAC38-9A and Nt-pSAC38-10C. The homoplastomic state was confirmed by DNA gel blot analysis.

15          **Nuclear-encoded plastid-targeted Cre genes.**

Plant activator lines in which *Ssu-tp-cre* is linked to a nuclear kanamycin resistance gene have been described in Example 1. The plastid marker to test CRE-activated inversion described in Example 2 utilizes a kanamycin resistance gene. Kanamycin resistance conferred by the plastid gene due to CRE-mediated inversion could not be distinguished from kanamycin resistance conferred by the marker gene of the Agrobacterium binary vector that was used to introduce the nuclear *cre*. Therefore, we have constructed activator strains in which *Ssu-tp-cre* is linked to gentamycin resistance. The *Ssu-tp22-cre* gene linked to the nuclear gentamycin resistance is the Cre3 strain and the *Ssu-tp5-cre* gene linked to gentamycin resistance is the Cre4 strain.

30          **Inversion of >*neo*< in the plastid genome of somatic cells.**

The nuclear *cre* genes were introduced into the

chloroplast >neo< tester strains by cocultivation of tobacco leaves with the Agrobacterium strains and selection for gentamycin resistance (100 mg/L).  
5 Digestion of total cellular DNA with BamHI and probing with the plastid targeting region (Apal-EcoRV fragment, Fig. 4) hybridizes to 1.8-kb and a 3.8-kb fragments in the parental Nt-pSAC38-10C lines (Fig. 10). Activation by CRE in lines Cre3-3 and Cre4-5 created a mixed population of >neo< genes representing the original and  
10 inverted orientations detected as the original 3.8-kb and 1.8-kb and the newly created 4.6-kb and 0.9-kb hybridizing fragments. Lines carrying the cre and an approximately wild-type size fragment are *aadA-neo* deletion derivatives, similar to those shown in Fig. 4.  
15 Thus, it appears that CRE mediated inversion via *lox* sites creates increased local recombination frequencies that leads to deletion of the transgenes via the short direct repeats of Prrn promoters.

20

**Controlling inversion via *lox* sites by CRE activity.**

Here we describe constructs for CRE-mediated inversion of plastid genome segments flanked by inverted *lox* sites. Inversion of the sequences is independent of the encoded genetic information and relies only on CRE activity. CRE activity may be provided transiently, by expression in plastids from plastid signals described in US patent 5,877,402, or from nuclear genes encoding a plastid-targeted CRE. Such plastid-targeted CRE constructs are described in Example 1, for example the *Ssu-tp5-cre* or *Sssu-tp22-cre* genes. Alternative approaches to provide CRE activity are stable incorporation of a plastid-targeted nuclear Cre into the nucleus of somatic (leaf) cells by Agrobacterium-mediated, PEG induced or biolistic

transformation or by fertilization with pollen from a transformed plant. The Agrobacterium P2 promoter and cauliflower mosaic virus 35S promoter exemplified here are constitutive promoters. Regulated expression of CRE may be important for certain applications.

Developmentally timed expression may be obtained from promoters with tissue specific activity. Regulated expression of CRE may be obtained from chemically induced nuclear gene promoters responding to elicitors, steroids, copper or tetracycline (reviewed in; Gatz et al. 1992; Mett et al. 1993; Aoyama and Chau 1997; Gatz 1997; Martinez et al. 1999; Love et al. 2000) and described in US patent 5,614,395.

#### 15      **Controlled expression of deleterious gene products**

There are a variety of valuable heterologous proteins that interfere with plastid metabolism. For example, certain proteins may be inserted into photosynthetic membranes and interfere with photosynthesis. This problem can be circumvented by first growing the plants to maturity, then activating production of the deleterious protein by chemically inducing CRE expression. CRE, in turn, will make the gene expressible by lox-mediated inversion of the coding region.

The molecular tools necessary for the construction of such plastid genes are described in present application. In case of the monocistronic inversion vector the gene of interest (goi) is flanked by inverted lox sites and is introduced by linkage with *aadA* (Fig. 12). The selectable marker (*aadA*) coding region is the first reading frame, and is expressed from the promoter. The *goi* reading frame is the second coding region, and it is not expressed as it is in an inverted orientation relative to the promoter. Expression of *goi* is induced

by CRE-mediated inversion of the *goi* coding region, as described for >*neo*< in Example 2 and is shown in Fig. 12.

The dicistronic lox inversion vector is shown in Fig. 13. In this case the inverted lox sites flank both *aadA* and *goi*. The selectable marker (*aadA*) coding region is expressed from the promoter. The *goi* reading frame is not expressed as it is in an inverted orientation relative to the promoter. Expression of *goi* is induced by CRE-mediated inversion of the *aadA-goi* containing region that results in simultaneous expression of *goi* and inactivation of *aadA*.

The presence of two *lox* sites may destabilize the plastid genome that leads to CRE-independent deletion of plastid genome sequences. However, it appears that CRE activity by itself is not mutagenic, and the plastid genomes are stable if only one *lox* site is present. Mutant *lox* sites that are efficiently excised but recombine into excision resistant sites have been described (Hoess et al. 1982; Albert et al. 1995). Such *lox* sites would mediate efficient inversion, but the new *lox* sites would be resistant to additional cycles of CRE activation. Providing only a short burst of CRE activation using a chemically induced promoter could further refine the expression system.

### EXAMPLE 3

#### CRE-MEDIATED DELETION TO OBTAIN MARKER FREE TRANSPLASTOMIC PLANTS AND FOR HIGH LEVEL EXPRESSION OF THE RECOMBINANT PROTEINS

Plastid *loxP* vectors in this section are described for CRE-mediated excision of selective markers in transplastomic plants. Since excision of sequences

between directly oriented *lox* sites is very efficient, variants of the same vectors can be used for CRE-activated expression of recombinant proteins. A family of plastid vectors with suitably positioned *lox* sites is shown schematically in Fig. 14 through Fig. 17.

The map of the basic tobacco plastid lox deletion vector is shown in Fig. 14. It contains (a) two directly oriented *lox* sites separated by a unique *Bgl*II cloning site and (b) an adjacent polycloning site. These sequences (Seq. ID No. 11) are inserted into the *Sca*I site plastid repeat vector pPRV100 (US Patent 5,877,402; Zoubenko et al. 1994). Suitable marker genes (*aadA*, *neo* or *kan*, *bar*, glyphosate resistance, bromoxynil resistance) for insertion into the *Bgl*II site have been described in US Patent 5,877,402, WO 00/07421 and WO 00/03022.

The map of the tobacco plastid *lox >aadA> deletion vector* is shown in Fig. 15. It is the basic *lox* deletion vector with an *aadA* gene cloned into the *Bgl*II sites oriented towards the *rrn* operon.

Maps of constitutive lox dicistronic deletion vectors are shown in Fig. 16 through Fig. 18. This dicistronic design enables simultaneous expression of both the first and the second open reading frames. The selectable marker designed for excision may be encoded in the first (Fig. 16) or second (Fig. 17, Fig. 18) open reading frames. Since a minimally 34 bp *lox* site is located between the two reading frames, both the marker gene (*aadA*) and the gene of interest have their own leader sequence to facilitate translation (Fig. 16, Fig. 17). Translational coupling may also be feasible if the *lox* site is incorporated in the marker gene coding region N terminus (Fig. 18). DNA sequence of promoter-*lox* constructs shown in Figs. 16 is set forth in Seq. ID

No. 1. Promoters and promoter-leader combinations suitable to promote high-level protein expression in plastids are described in European Patent Applications WO 00/07421, WO 97/06250 and WO 98/55595. Sequences 5 suitable for directly oriented lox sites are given in Seq. ID No. 11. Translational coupling between a gene of interest and the downstream *aadA* is shown in Fig. 18. There are multiple ways of achieving translational coupling between adjacent genes (Baneyx 1999). One 10 approach is incorporation of a properly spaced ribosome binding-site in the upstream gene's coding region (Schoner et al. 1986; Omer et al. 1995). An example for a suitable sequence directly upstream of the translation initiation codon (ATG) would be G-GAG-GAA-TAA-CTT-ATG. 15 A specific example for the use of the sequence is translational coupling between a *bar* (suitable source described in European Patent Application WO 00/07421) and a downstream *aadA* are given in Seq. ID No. 12. Note SalI site downstream of AUG incorporated to facilitate 20 engineering the BglII-SalI region and the directly oriented lox sites in the *aadA* coding region and downstream of *aadA*. The sequence is given for a BglII-SpeI fragment. The BglII site is within the *bar* coding region; the SpeI site is downstream of the second lox 25 site, as marked in Fig. 18. If a C-terminal extension to create a ribosome binding site is unacceptable, a suitable sequence may be obtained by silent mutagenesis of the coding region at the third codon position. Variants of plastid ribosome binding sites have been 30 catalogued (Bonham-Smith and Bourque 1989)

A tobacco inducible lox deletion vector is shown in Fig. 19. The marker gene (*aadA*) is encoded in the first reading frame, followed by a silent *goi* lacking the translation initiation codon (ATG) and the 5'

untranslated leader. Expression of the *goi* frame is triggered by *aadA* excision that results in translational fusion of the *aadA* N-terminal region with the *goi*. After *aadA* excision the *goi* mRNA is translated from the *aadA* translation control signals, the 5' UTR and AUG. DNA sequence of the SacI-NheI fragment is given in Seq. ID. No. 13. The Prrn promoter-*atpB* translational control region is described in European Patent Application WO 00/07421. The *aadA* construct has two directly-oriented lox sites: one in the coding region N-terminus and one downstream of *aadA* to facilitate CRE-mediated excision of the marker gene.

#### EXAMPLE 4

##### 15 DELETION OF VITAL PLASTID GENES TO OBTAIN CYTOPLASMIC MALE STERILITY

US Patent 5,530,191 provides a cytoplasmic male sterility (CMS) system for plants, which is based on modification of the plastid genome. The CMS system comprises three transgenes: a "plastid male sterility" gene that causes plastid and cellular disablement of the anther tissue, and two nuclear genes that regulate the expression of the plastid gene. An important feature of the system is developmentally timed cellular death based on the expression, or the lack of the expression, of a plastid gene. As one specific approach to induce developmentally timed ablation of anther tissue we describe CRE-mediated excision of essential plastid genes via directly oriented lox sites.

30 The number of genes encoded by the plastid genome is about 120. Some of the genes are non-essential and may be inactivated by targeted gene disruption without a major phenotypic consequence. Good examples are the plastid *ndh* genes (Burrows et al. 1998; Shikanai et al.

1998) or the *trnV* gene the deletion of which has been described in Example 1. Excision of these genes is unlikely to cause cell ablation. The photosynthetic genes are essential for survival under field conditions.

5 However, pigment deficient, non-photosynthetic plants can be maintained as long as they are grown on a sucrose-containing medium, or are grafted onto photosynthetically active wild-type (green) plants (Kanevski and Maliga 1994). Some of the house-keeping

10 genes, such as the genes encoding the plastid multisubunit RNA polymerase are essential for photosynthetic growth, but not for survival (Allison et al. 1996). Thus, deletion of these genes is not suitable to trigger cell death. Only a relatively small number of

15 plastid genes have proven to be essential for viability. The essential nature of the genes was recognized by the lack of homoplasmic cells in gene disruption experiments indicating that the loss of these genes results in cellular death. Cellular death due to lack of

20 plastid function is understandable, as plastids are the site of the biosynthesis of amino acids, several lipids and are required for nitrate assimilation. Examples of

25 plastid genes essential for cellular survival are the *clpP* protease subunit gene (Huang et al. 1994), *ycf1* and *ycf2*, the two largest plastid-encoded open reading frames (Drescher et al. 2000).

To induce cellular death by CRE-mediated excision, directly oriented *lox* sites can be incorporated in the plastid genome flanking essential genes, as shown for *clpP* in Fig. 20. The *clpP* gene has two large introns (807 bp and 637 bp) and the region can be conveniently cloned as a SalI-SphI fragment. The selectable marker *aadA* is inserted into a *KpnI* restriction site created by PCR mutagenesis downstream of *clpP* Exon 3, oriented towards *rps12* Exon I. One of the *lox* sites is

engineered next to the *aadA* gene, the second *lox* site is inserted in Intron I. Cellular death is induced by activation of the nuclear *Cre* gene as described in US Patent 5,530,191. It is necessary to use a selective marker, such as *aadA* to introduce the *lox* sites into the plastid genome. The *aadA* gene can subsequently eliminated using a second, independent site specific recombinase such as FRT via the *frt* sites engineered into the transformation vector shown in Fig. 20.

Alternative targets for CRE-mediated deletion in a CMS system are the essential ribosomal protein genes such as *rpl23*, the ribosomal RNA operon (for insertion sites see; Staub and Maliga 1992; Zoubenko et al. 1994) and the *ycf1* and *ycf2* genes (Drescher et al. 2000)

The following sequences are referred to throughout the specification and facilitate the practice of the present invention.

SEQ. No. 1: PrrnloxI. sequence

gagctcGCTCCCCGCCGTCAATGAGAATGGATAAGAGGCTCGTGGGATTGA  
CGTGAGGGGGCAGGGATGGCTATATTCTGGGAGCataacttcgtataatgtatgc  
tatacgaagttagtctaga

SEQ. No. 2: TrbcLloxI. sequence

gaattcataacttcgtatacgtacatacattatacgaaggtaAGACATTAGCAGATAA  
ATTAGCAGGAAATAAAGAAGGATAAGGAGAAAGAACTCAAGTAATTATCCTTCGTT  
CTCTTAATTGAATTGCAATTAAACTCGGCCAATCTTTACTAAAAGGATTGAGCC  
GAATAACAACAAAGATTCTATTGCATATATTGACTAAGTATATACTTACCTAGAT  
ATACAAAGATTGAAATACAAAATCTAGcaagcttggtacc

SEQ. No. 3: cre coding region. sequence

35 gagctccATGgctagcTCC AATTACTGA CCGTACACCA AAATTGCGCT  
GCATTACCGG TCGATGCAAC GAGTGATGAG GTTCGCAAGA ACCTGATGGA  
CATGTTCAAGG GATGCCAGG CGTTTCTGA GCATACCTGG AAAATGCTTC  
TGTCCGTTTG CCGGTCGTGG GCAGGATGGT GCAAGTTGAA TAACCGGAAA  
40 TGGTTTCCCG CAGAACCTGA AGATGTTCGC GATTATCTTC TATATCTTC  
GGCGCGCGGT CTGGCAGTAA AACTATCCA GCAACATTTG GGCCAGCTAA  
ACATGCTTCA TCGTCGGTCC GGGCTGCCAC GACCAAGTGA CAGCAATGCT  
GTTTCACTGG TTATGCGGGCG GATCCGAAAA GAAAACGTTG ATGCCGGTGA

ACGTGCAAAA CAGGCTCTAG CGTTCGAACG CACTGATTTC GACCAGGTT  
 GTTCACTCAT GGAAAATAGC GATCGCTGCC AGGATATAACG TAATCTGGCA  
 TTTCTGGGGA TTGCTTATAA CACCCCTGTTA CGTATAGCCG AAATTGCCAG  
 GATCAGGGTT AAAGATATCT CACGTACTGA CGGTGGGAGA ATGTTAATCC  
 5 ATATTGGCAG AACGAAAACG CTGGTTAGCA CCGCAGGTGT AGAGAAGGCA  
 CTTAGCCTGG GGGTAACTAA ACTGGTCGAG CGATGGATT CCGTCTCTGG  
 TGTAGCTGAT GATCCGAATA ACTACCTGTT TTGCCGGGTC AGAAAAAATG  
 GTGTTGCCGC GCCATCTGCC ACCAGCCAGC TATCAAATCG CGCCCTGGAA  
 GGGATTTTG AAGCAACTCA TCGATTGATT TACGGCGCTA AGGATGACTC  
 10 TGGTCAGAGA TACCTGGCCT GGTCTGGACA CAGTGGCCGT GTCGGAGCCG  
 CGCGAGATAT GGCCCGCGCT GGAGTTCAA TACCGGAGAT CATGCAAGCT  
 GGTGGCTGGA CCAATGTAAA TATTGTCATG AACTATATCC GTAACCTGGA  
 TAGTGAAACA GGGGCAATGG TGCGCCTGCT cGAGGATGGC GATTAGtctaga

15

## SEQ. No. 4: PrrnloxD. Sequence

gagctcGCTCCCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGATTGA  
 CGTGAGGGGGCAGGGATGGCTATATTCTGGGAGCataacttcgtataatgtatgc  
 20 tatacgaagttatgaattc

## SEQ. No. 5: TrbcLloxD. sequence

tctagataacttcgtataatgtatgctatacgaagttatAGACATTAGCAGATAAA  
 25 TTAGCAGGAAATAAAGAAGGATAAGGAGAAAGAACTCAAGTAATTATCCTTCGTT  
 TCTTAATTGAATTGCAATTAAACTCGGCCAATCTTTACTAAAAGGATTGAGCCG  
 AATACAACAAAGATTCTATTGCATATATTTGACTAAGTATATACTTACCTAGATA  
 TACAAGATTGAAATACAAATCTAGcaagttggtaacc

30

## SEQ. No. 6: Pea ssuTP5. sequence

ccggatccAA TTCAACCACA AGAACTAACAA AAGTCAGAAA AATGGCTTCT  
 ATGATATCCT CTCGCTGT GACAAACAGTC AGCCGTGCTT CTAGGGTGCA  
 35 ATCCGCGGCA GTGGCTCCAT TCAGCGGCCT GAAATCCATG ACTGGATTCC  
 CAGTGAAGAA GGTCAACACT GACATTACTT CCATTACAAG CAATGGTGGA  
 AGAGTAAAGT GCATGCAGGT GTGGCCTGCC atggcttagc

40

## SEQ. No. 7: Pea ssuTP22. sequence

ccggatcc AA TTCAACCACA AGAACTAACAA AAGTCAGAAA AATGGCTTCT  
 ATGATATCCT CTCGCTGT GACAAACAGTC AGCCGTGCTT CTAGGGTGCA  
 45 ATCCGCGGCA GTGGCTCCAT TCAGCGGCCT GAAATCCATG ACTGGATTCC  
 CAGTGAAGAA GGTCAACACT GACATTACTT CCATTACAAG CAATGGTGGA  
 AGAGTAAAGT GCATGCAGGT GTGGCCTCCA ATTGGAAAGA AGAAGTTGA  
 GACTCTTCCT TATTGCCCCAT CATGACCAat ggcttagc

50

## SEQ. No. 8: Pea ssuTP23. sequence

5           ccggatccAA TTCAACCACA AGAACTAAC AAGTCAGAAA AATGGCTTCT  
 ATGATATCCT CTTCCGCTGT GACAACAGTC AGCCGTGCTT CTAGGGTGCA  
 ATCCCGGGCA GTGGCTCCAT TCGGGCGCCT GAAATCCATG ACTGGATTCC  
 CAGTGAAGAA GGTCAACACT GACATTACTT CCATTACAAG CAATGGTGGA  
 AGAGTAAAGT GCATGCAGGT GTGGCCTCCA ATTGGAAAGA AGAAGTTTGA  
 GACTCTTCC TATTGCCAC CATTGACCAg AGATCAGTTG gctagcgg

## SEQ. No. 9: P2 promoter sequence

10          gaattCATT TCACGTGTGG AAGATATGAA TTTTTTGAG AAACTAGATA  
 AGATTAATGA ATATCGGTGT TTTGGTTTT TCTTGTGGCC GTCTTGTTT  
 ATATTGAGAT TTTTCAAATC AGTGCAGCAAG ACGTGACGTA AGTATCTGAG  
 CTAGTTTTA TTTTCTACT AATTGGTCG TTTATTCGG CGTGTAGGAC  
 ATGGCAACCG GGCCTGAATT TCGCGGGTAT TCTGTTCTA TTCCAACCTT  
 15          TTCTTGATCC GCAGCCATTA ACGACTTTG AATAGATACG CTGACACGCC  
 AAGCCTCGCT AGTCAAAGT GTACCAAACA ACGCTTTACA GCAAGAACGG  
 AATGCGCGTG ACGCTCGCG TGACGCCATT TCGCCTTTTC AGAAATGGAT  
 AAATAGCCTT GCTTCCTATT ATATCTTCCC AAATTACCAA TACATTACAC  
 TAGCATCTGA ATTCATAAC CAATCTCGAT ACACCAAATC GATaggatcc  
 20          taccatgg

## SEQ. No. 10: 35S promoter sequence

25          AAGCTTGCCA ACATGGTGGA GCACGACACT CTCGTCTACT CCAAGAATAT  
 CAAAGATACA GTCTCAGAAG ACCAAAGGGC TATTGAGACT TTTCAACAAA  
 GGGTAATATC GGGAAACCTC CTCGGATTCC ATTGCCAGC TATCTGTCAC  
 TTCATCAAAA GGACAGTAGA AAAGGAAGGT GGCACCTACA AATGCCATCA  
 TTGCGATAAA GGAAAGGCTA TCGTTCAAGA TGCCTCTGCC GACAGTGGTC  
 CAAAGATGG ACCCCCCACCC ACGAGGAGCA TCGTGGAAAA AGAAGACGTT  
 30          CCAACCACGT CTTCAAAGCA AGTGGATTGA TGTGATAACA TGGTGGAGCA  
 CGACACTCTC GTCTACTCCA AGAATATCAA AGATACAGTC TCAGAAGACC  
 AAAGGGCTAT TGAGACTTT CAACAAAGGG TAATATCGGG AACACCTCCTC  
 GGATTCCATT GCCCAGCTAT CTGTCACTTC ATCAAAGGA CAGTAGAAAA  
 35          GGAAGGTGGC ACCTACAAAT GCCATCATTG CGATAAAGGA AAGGCTATCG  
 TTCAAGATGC CTCTGCCGAC AGTGGTCCC AAGATGGACC CCCACCCACG  
 AGGAGCATCG TGGAAAAGA AGACGTTCCA ACCACGTCTT CAAAGCAAGT  
 GGATTGATGT GATATCTCCA CTGACGTAAG GGATGACGCA CAATCCCAC  
 ATCCTTCGCA AGACCCCTCC TCTATATAAG GAAGTTCATT TCATTGGAG  
 40          AGGACACGCT GAAATCACCA GTCTCTCT ACAAAATCTAT CTCTCTCGAT  
 TCGCGAGCTC GGTACCCGGG gatcgatcc

## SEQ. No. 11: KpnI-lox-BglII-lox-HindIII fragment

45          ggtaaccATAACTTCGTATAATGTATGCTATACGAAGTTAGatctATAACTTCGT  
 ATAATGTATGCTATACGAAGTTATAagctt

50          Seq. ID No. 12. Translational coupling of bar and aadA  
 according to scheme in Fig. 18. BglII-SpeI fragment.

GAGATCTGgg aggaataact tATGgggtc gacATAACTT CGTATAATGT  
 ATGCTATACG AAGTTATTaG AAGCGGTGAT CGCCGAAGTA TCGACTCAAC

5            TATCAGAGGT AGTTGGCGTC ATCGAGCGCC ATCTCGAACCC GACGTTGCTG  
           GCCGTACATT TGTACGGCTC CGCAGTGGAT GGCAGGCTGA AGCCACACAG  
           TGATATTGAT TTGCTGGTTA CGGTGACCCT AAGGCTTGAT GAAACAACGC  
           GGCAGAGCTT GATCAACGAC CTTTGGAAA CTTCGGCTTC CCCTGGAGAG  
           AGCGAGATTC TCCCGCCTGT AGAAGTCACC ATTGTTGTGC ACGACGACAT  
           CATTCCGTGG CGTTATCCAG CTAAGCGCGA ACTGCAATTG GGAGAATGGC  
           AGCGCAATGA CATTCTTGCA GGTATCTTCG AGCCAGGCCAC GATCGACATT  
           GATCTGGCTA TCTTGCTGAC AAAAGCAAGA GAACATAGCG TTGCCTGGT  
           AGGTCCAGCG GCGGAGGAAC TCTTGATCC GGTTCTGAA CAGGATCTAT  
           TTGAGGCCTA AAATGAAACC TTAACGCTAT GGAACCTCGCC GCCCGACTGG  
           GCTGGCGATG AGCGAAATGT AGTGCCTTACG TTGTCCTCGCA TTTGGTACAG  
           CGCAGTAACC GGCAAATCG CGCCGAAGGA TGTCGCTGCC GACTGGGCAA  
           TGGAGCGCCT GCCGGCCCAG TATCAGCCCCG TCATACTTGA AGCTAGACAG  
           GCTTATCTTG GACAAGAAGA AGATCGCTTG GCCTCGCGCG CAGATCAGTT  
           GGAAGAATTG GTCCACTACG TGAAAGGCAG GATCACCAAG GTAGTCGGCA  
           AATAAATAAC TTCGTATAAT GTATGCTATA CGAAGTTATA ctagt

20            Seq. ID No. 13. CRE-induced expression of recombinant protein according to design in Fig. 19. SacI-NheI fragment.

25            gagctcGCTC CCCCGCCGTC GTTCAATGAG AATGGATAAG AGGCTCGTGG  
           GATTGACGTG AGGGGGCAGG GATGGCTATA TTTCTGGAG AATTAACCGA  
           TCGACGTGCa AGCGGACATT TATTTCATTAAT TCGATAATTG TTGCAAAAC  
           ATTTCGACAT ATTATTTTAT TTTATTATTA TGgggATAAC TTCTGATAAT  
           GTATGCTATA CGAAGTTATT aGAAGCGGTG ATCGCCGAAG TATCGACTCA  
           ACTATCAGAG GTAGTTGGCG TCATCGAGCG CCATCTCGAA CCGACGTTGC  
           TGGCCGTACA TTTGTACGGC TCCGCAGTGG ATGGCGGCCT GAAGCCACAC  
           AGTGTATTTG ATTGCTGGT TACGGTGACC GTAAGGCTTG ATGAAACAAAC  
           30        GCGGCGAGCT TTGATCAACG ACCTTTGGA AACTTCGGCT TCCCCTGGAG  
           AGAGCGAGAT TCTCCGCGCT GTAGAAGTCA CCATTGTTGT GCACGACGAC  
           ATCATTCCGT GGCATTATCC AGCTAAGCGC GAACTGCAAT TTGGAGAATG  
           GCAGCGCAAT GACATTCTTG CAGGTATCTT CGAGCCAGCC ACGATCGACA  
           TTGATCTGGC TATCTGCTG ACAAAAGCAA GAGAACATAG CGTTGCCTTG  
           35        GTAGGTCCAG CGGGGGAGGA ACTCTTGAT CCGGTTCTG AACAGGATCT  
           ATTGAGGCG CTAAATGAAA CCTTAACGCT ATGGAACCTCG CCGCCCGACT  
           GGGCTGGCGA TGAGCGAAAT GTAGTCTTA CGTTGTCCCG CATTGGTAC  
           AGCGCAGTAA CCGGCAAAAT CGCGCCGAAG GATGTCGCTG CCGACTGGGC  
           AATGGAGCGC CTGCCGGCCC AGTATCAGCC CGTCATACTT GAAGCTAGAC  
           40        AGGCTTATCT TGGACAAGAA GAAGATCGCT TGGCCTCGCG CGCAGATCAG  
           TTGGAAGAAT TTGTCCACTA CGTGAAAGGC GAGATCACCA AGGTAGTCGG  
           CAAATAAATA ACTTCGTATA ATGTATGCTA TACGAAGTTA Ttagctagc

45

**REFERENCES**

Adams DE, Bliska JB, Cozzarelli NR (1992) Cre-lox recombination in Escherichia coli cells. Mechanistic differences from the in vitro reaction. J Mol Biol 226:661-673

- Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J* 7:649-659
- 5 Allison LA, Simon LD, Maliga P (1996) Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. *EMBO J* 15:2802-2809
- 10 Aoyama T, Chau NH (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J* 11:605-612
- Baneyx F (1999) Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol* 10:411-421
- 15 Beck CF, Ingraham JL, Neuhardt J, Thomassen E (1972) Metabolism of pyrimidines and pyrimidine nucleosides by *Salmonella typhimurium*. *J Bacteriol* 110:219-228
- 20 Bonham-Smith PC, Bourque DP (1989) Translation of chloroplast-encoded mRNA: potential initiation and termination signals. *Nucleic Acids Res* 17:2057-2080
- Burrows PA, Sazanov LA, Svab Z, Maliga P, Nixon PJ (1998) Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid *ndh* genes. *EMBO J* 17:868-876
- 25 Carrer H, Hockenberry TN, Svab Z, Maliga P (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Mol Gen Genet* 241:49-56
- 30 Carrer H, Maliga P (1995) Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. *Biotechnology* 13:791-794
- 35 Carrer H, Staub JM, Maliga P (1990) Gentamycin resistance in *Nicotiana* conferred by AAC(3)-I, a narrow substrate specificity acetyl transferase. *Plant Mol Biol* 17:301-303
- 40 Craig NL (1988) The mechanism of conservative site-specific recombination. *Annual Review Of Genetics* 22:77-105
- 45 Dale EC, Ow DW (1991) Gene transfer with subsequent removal of the selection gene from the host genome. *Proc Natl Acad Sci USA* 88:10558-10562

- Depicker AG, Jacobs AM, Montagu MC (1988) A negative selection scheme for tobacco protoplast-derived cells expressing the T-DNA gene 2. *Plant Cell Rep* 7:63-66
- 5 Drescher A, Ruf S, Calsa T, Carrer H, Bock R (2000) The two largest chloroplast genome-encoded open reading frames of higher plants are essential genes. *The Plant Journal* 22:97-104
- 10 Dršge M, Přhler A, Selbitschka W (1998) Horizontal gene transfer as a biosafety issue: a natural phenomenon of public concern. *J Biotechnol* 64:75-90  
Gatz C (1997) Chemical control of gene expression. *Ann Rev Plant Physiol Plant Mol Biol* 48:89-108
- 15 Gatz C, Frohberg C, Wendenburg R (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant J* 2:397-404
- 20 Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant Mol Biol* 25:989-994
- 25 Hoess RH, Ziese M, Sternberg N (1982) P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc Natl Acad Sci USA* 79:3398-3402
- 30 Huang C, Wang S, Chen L, Lemioux C, Otis C, Turmel M, Liu XQ (1994) The *Chlamydomonas* chloroplast *clpP* gene contains translated large insertion sequences and is essential for cell growth. *Molecular and Genetal Genetics* 244:151-159
- 35 Kanevski I, Maliga P (1994) Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proc Natl Acad Sci USA* 91:1969-1973
- 40 Karlin-Neumann GA, Brusslan JA, Tobin EM (1991) Phytochrome control of the *tms2* gene in transgenic *Arabidopsis*: a strategy for selecting mutants in the signal transduction pathway. *Plant Cell* 3:573-582
- 45 Khan MS, Maliga P (1999) Fluorescent antibiotic resistance marker to track plastid transformation in higher plants. *Nat Biotechnol* 17:910-915
- 50 Kolb AF, Siddell SG (1996) Genomic targeting with an MBP-Cre fusion protein [published erratum appears in Gene 1997 Apr 11;189(1):149]. *gene* 183:53-60

- Le Y, Gagneten S, Tombaccini D, Bethke B, Sauer B (1999) Nuclear targeting determinants of the phage P1 Cre DNA recombinase. *Nucleic Acids Res* 27:4703-4709
- 5 Lichtenstein C, Barrena E (1993) Prospects for reverse genetics in plants using recombination [news]. *Plant Mol Biol* 21:v-xii
- 10 Love J, Scott AC, Thompson WF (2000) Stringent control of transgene expression in *Arabidopsis thaliana* using the Top10 promoter system. *Plant J* 21:579-588
- 15 Lubben TH, Gatenby AA, Ahlquist P, Keegstra K (1989) Chloroplast import characteristics of chimeric proteins. *Plant Mol Biol* 12:13-18
- 20 Lyznik LA, Hirayama L, Rao KV, Abad A, Hodges TK (1995) Heat-inducible expression of FLP gene in maize cells. *Plant J* 8:177-186
- 25 Lyznik LA, Mitchell JC, Hirayama L, Hodges TK (1993) Activity of yeast FLP recombinase in maize and rice protoplasts. *Nucleic Acids Res* 21:969-975
- 30 Lyznik LA, Rao KV, Hodges TK (1996) FLP-mediated recombination of FRT sites in the maize genome. *Nucleic Acids Res* 24:3784-3789
- 35 Maliga P (1993) Towards plastid transformation in higher plants. *Trends Biotech* 11:101-107
- Martinez A, Sparks C, Hart CA, Thompson J, Jepson I (1999) Ecdysone agonist inducible transcription in transgenic tobacco plants. *Plant J* 19:97-106
- 40 Morris AC, Schaub TL, James AA (1991) FLP-mediated recombination in the vector mosquito, *Aedes aegypti*. *Nucleic Acids Res* 19:5895-5900
- 45 Nussaume L, Vincentz M, Caboche M (1991) Constitutive nitrate reductase: a dominant conditional marker for plant genetics. *Plant J* 1:267-274
- 50 O'Gorman S, Fox DT, Wahl GM (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251:1351-1355

- Omer CA, Diehl RE, Kral AM (1995) Bacterial expression and purification of human protein prenyltransferases using epitope-tagged, translationally coupled systems. *Meth Enzymol* 250:3-12  
5
- Perera RJ, Linard CG, Signer ER (1993) Cytosine deaminase as a negative selective marker for *Arabidopsis*. *Plant Mol Biol* 23:793-799
- Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. *Mol Gen Genet* 234:49-59  
10
- Schoner BE, Belagaje RM, Schoner RG (1986) Translatrion of a synthetic two-cidstron mRNA in *Escherichia coli*. *Proc Natl Acad Sci USA* 83:8506-8510  
15
- Serino G, Maliga P (1997) A negative selection scheme based on the expression of cytosine deaminase in plastids. *Plant J* 12:697-701  
20
- Shikanai T, Endo T, Hashimoto T, Yamada Y, Asada K, Yokota A (1998) Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. *Proc Natl Acad Sci USA* 95:9705-9709  
25
- Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsabayashi T, Zaita N, Chungwongse J, Obokata J, Yamaguchi-Shinozaki K, Deno H, Kamogashira T, Yamada K, Kasuda J, Takaiwa F, Kato A, Todoh N, Shimada H, Sugiura M (1986) The complete sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J* 5:2043-2049  
30
- Small I, Wintz H, Akashi K, Mireau H (1998) Two birds with one stone: genes that encode products targeted to two or more compartments. *Plant Mol Biol* 38:265-277  
35
- Soll J, Tien R (1998) Protein translocation into and across the chloroplastic envelope membranes. *Plant Mol Biol* 38:191-207  
40
- Sriraman P (2000) Identification and characterization of components of the plastid transcription machinery. Identification and characterization of components of the plastid transcription machinery. Rutgers University, Piscataway, NJ  
45
- Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated trough the resolution of complex integration patterns. *Proceedings of the National Academy of Sciences* 96:11117-11121  
50

Staub JM, Maliga P (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. *Plant Cell* 4:39-45

5 Staub JM, Maliga P (1995) Expression of a chimeric uidA gene indicates that polycistronic mRNAs are efficiently translated in tobacco plastids. *Plant J* 7:845-848

10 Stougaard J (1993) Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. *Plant J* 3:755-761

15 Stricklett PK, Nelson RD, Kohan DE (1998) Site-specific recombination using an epitope tagged bacteriophage P1 Cre recombinase. *gene* 215:415-423

20 Sundaresan V, Springer P, Volpe T, Haward S, Jones JD, Dean C, Ma H, Martienssen R (1995) Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev* 9:1797-1810

25 Svab Z, Harper EC, Jones JD, Maliga P (1990) Aminoglycoside-3''-adenyltransferase confers resistance to spectinomycin and streptomycin in *Nicotiana tabacum*. *Plant Mol Biol* 14:197-205

30 Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc Natl Acad Sci USA* 90:913-917

Sylvanen M (1999) In search of horizontal gene transfer. *Nat Biotechnol* 17:833

35 Tepfer D (1989) Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizosphere biology and plant development, ecology and evolution. In: Kosuge T, Nester EW (eds) *Plant-Microbe Interactions. Molecular and Genetic Perspectives*, Vol. 3, McGraw-Hill, New York, pp 294-342

40 Timko MP, Kaush AP, Hand JM, Cashmore AR (1985) Structure and expression of nuclear genes encoding polypeptides of the photosynthetic apparatus. In: Steinback KE, Bonitz S, Arntzen CJ, Bogorad L (eds) *Molecular biology of the photosynthetic apparatus*, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp 381-396.

45 50 Timmermans MCP, Maliga P, Vieira J, Messing J (1990) The pFF plasmids: cassettes utilizing CaMV sequences for

expression of foreign genes in plants. *J Biotechnol* 14:333-344.

5 van Haaren MJ, Ow DW (1993) Prospects of applying a combination of DNA transposition and site-specific recombination in plants: a strategy for gene identification and cloning. *Plant Mol Biol* 23:525-533

10 Velten J, Velten L, Hain R, Schell J (1984) Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J* 3:2723-2730.

15 Wasmann CC, Reiss B, Bartlett SG, Bohnert HJ (1986) The import of the transit peptide and the transport protein for protein import into chloroplasts. *Mol Gen Genet* 205:446-453

20 Wimmer B, LotteSpeich F, van der Klei I, Veenhuis M, Gietl C (1997) The glyoxysomal and plastid molecular chaperones (70-kDa heat shock protein) of watermelon cotyledons are encoded by a single gene. *Proc Natl Acad Sci USA* 94:13624-13629

25 Xiang C, Guerra DJ (1993) The anti-nptII gene. A potential negative selective marker for plants. *Plant Physiol* 102:287-293

30 Zoubenko OV, Allison LA, Svab Z, Maliga P (1994) Efficient targeting of foreign genes into the tobacco plastid genome. *Nucleic Acids Res* 22:3819-3824

35 While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A site specific recombination method for  
5 removal of predetermined nucleic acid sequences from the  
plastid genome, said method comprising:

10 a) providing a first nucleic acid construct,  
said construct comprising a promoter being operably  
linked to a nucleic acid encoding an optional plastid  
targeting transit sequence which is operably linked to a  
nucleic acid encoding a protein having excision  
activity, said construct further comprising a first  
selectable marker encoding nucleic acid having plant  
specific 5' and 3' regulatory nucleic acid sequences;

15 b) providing a second DNA construct, said  
second construct comprising an second selectable marker  
encoding nucleic acid and excision sites, said second  
construct optionally containing a gene of interest, said second  
construct further comprising flanking plastid  
20 targeting nucleic acid sequences which facilitate  
homologous recombination into said plastid genome;

c) introducing said second DNA construct into  
a plant cell;

25 d) culturing said plant cell of step c) in the  
presence of a selection agent, thereby selecting for  
those plant cells expressing the proteins encoded by  
said second DNA construct;

30 e) introducing said first DNA construct into  
plant cells from step d) in the presence of a selection  
agent and selecting those plant cells expressing  
proteins encoded by said first construct, which when  
present said excising activity acts on said excision  
sites, thereby excising said predetermined target  
sequence.

2. A method as claimed in claim 1, wherein a plant is regenerated from plant cells of step c), cells are then contacted with said first construct and steps d) and e) are performed.

5

3. A method as claimed in claim 1, wherein said first construct is that depicted in Figure 3.

4. A method as claimed in claim 1, wherein said  
10 second construct is that depicted in Figure 2.

15 encoded integrase, and transposase.

protein having excision activity is selected from the group consisting of CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

30

7. A method as claimed in claim 1, wherein said selection agent is selected from the group consisting of kanamycin, gentamycin, spectinomycin, streptomycin and hygromycin, phosphinotricin, basta, glyphosate and bromoxynil.

25

8. A method as claimed in claim 1, wherein said excision of said predetermined sequence creates an expressible translational fusion protein.

30

10. A method as claimed in claim 1, wherein said predetermined target sequence is the selectable marker encoding nucleic acid present in said second construct.

11. A plant regenerated from the method of claim 1.

35

12. A site specific recombination system comprising the constructs of claim 1.

13. A site specific recombination method for removal of predetermined nucleic acid sequences from the plastid genome, said method comprising:

a) providing a first nucleic acid construct, said construct comprising a regulated promoter being operably linked to a nucleic acid encoding an optional plastid targeting transit sequence which is operably linked to a nucleic acid encoding a protein having excision activity, said construct optionally further comprising a first selectable marker encoding nucleic acid having plant specific 5' and 3' regulatory nucleic acid sequences;

b) providing a second DNA construct, said second construct comprising an second selectable marker encoding nucleic acid and excision sites, said second construct further comprising flanking plastid targeting nucleic acid sequences which facilitate homologous recombination into said plastid genome at a predetermined target sequence such that excision sites flank said predetermined target sequence following homologous recombination;

c) introducing said second DNA construct into a plant cell;

d) culturing a plant cell of step c) in the presence of a selection agent, thereby selecting for those plant cells expressing the proteins encoded by said second DNA construct;

e) regenerating a plant from cells obtained in step d);

f) introducing said first DNA construct into plant cells from step e) in the presence of a selection agent and selecting those plant cells expressing

proteins encoded by said first construct, which when present said excising activity acts on said excision sites, thereby excising said predetermined target sequence.

5

14. A method as claimed in claim 13, wherein said regulatable promoter is selected from the group of promoters consisting of inducible promoters, tissue specific promoters, developmentally regulated promoters and chemically inducible promoters.

10

15. A method as claimed in claim 13, wherein said predetermined target sequence is selected from the group consisting of genes associated with male sterility, clpP ribosomal proteins, ribosomal RNA operon sequences.

15

16. A method as claimed in claim 13, wherein said protein having excision activity is selected from the group consisting of CRE, flippase, resolvase, FLP, SSV1-encoded integrase, and transposase.

20

17. A method as claimed in claim 13, wherein said excision sites are LOX sequences, and *frt* sequences.

25

18. A method as claimed in claim 13, wherein said selection agent is selected from the group consisting of kanamycin, gentamycin, spectinomycin, streptomycin and hygromycin, phosphinotricin, basta, glyphosate and bromoxynil.

30

19. A plant regenerated from the method of claim 13.

20. A site specific recombination system for removal of predetermined nucleic acid sequences comprising the construct of claim 13.

5 21. Progeny plants obtained from the plant of claim 11.

22. Progeny plants obtained from the plant of claim 18.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
29 March 2001 (29.03.2001)

PCT

(10) International Publication Number  
**WO 01/21768 A1**

(51) International Patent Classification<sup>7</sup>: **C12N 5/04**,  
15/09, 15/63, 15/64, 15/65, 15/67, 15/82, 15/87, 15/90,  
A01H 1/00, 5/00

Street, Apt. A, Highland Park, NJ 08904 (US). **LUTZ,**  
**Kerry** [US/US]; 8 Liberty Place, Jackson, NJ 08527 (US).

(21) International Application Number: PCT/US00/25930

(74) Agents: **RIGAUT, Kathleen, D.** et al.; Dann, Dorfman,  
Herrell and Skillman, Suite 720, 1601 Market Street,  
Philadelphia, PA 19120 (US).

(22) International Filing Date:  
21 September 2000 (21.09.2000)

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(30) Priority Data:  
60/155,007 21 September 1999 (21.09.1999) US  
60/211,139 13 June 2000 (13.06.2000) US

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): **RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY**  
[US/US]; Old Queens, Somerset Street, New Brunswick,  
NJ 08903 (US).

**Published:**

— *With international search report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(72) Inventors; and

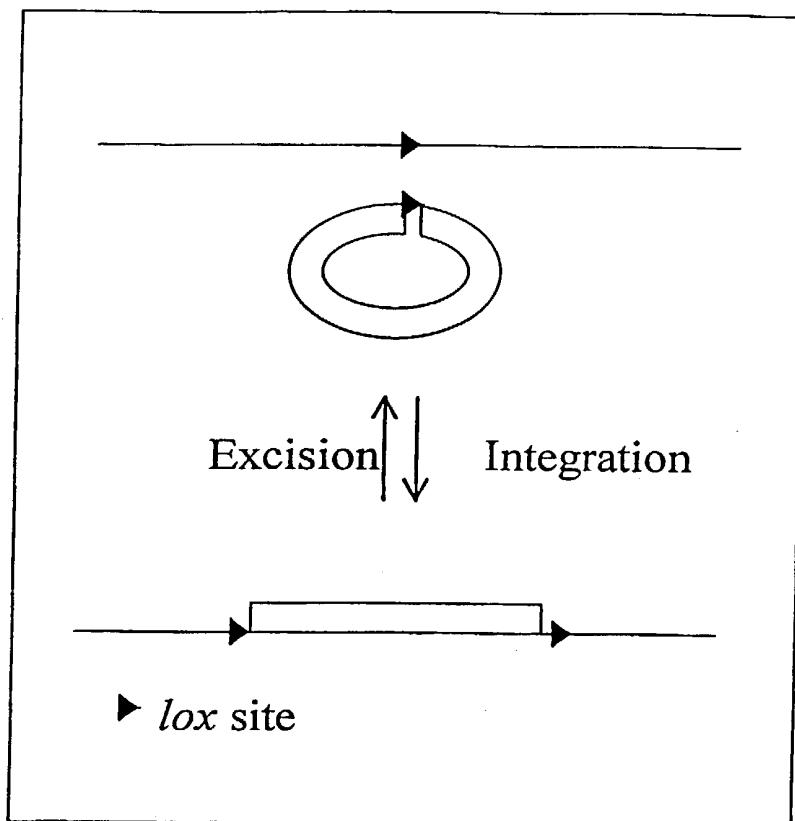
(75) Inventors/Applicants (*for US only*): **MALIGA, Pal**  
[HU/US]; 72 Yorktown Road, East Brunswick, NJ 08816  
(US). **CORNEILLE, Sylvie** [FR/US]; 305 Magnolia

WO 01/21768 A1

(54) Title: SITE-SPECIFIC RECOMBINATION SYSTEM TO MANIPULATE THE PLASTID GENOME OF HIGHER PLANTS

(57) Abstract: A site specific recombination system and methods of use thereof are disclosed for manipulating the genome of higher plants.

1/20



**Figure 1**

SUBSTITUTE SHEET (RULE 26)

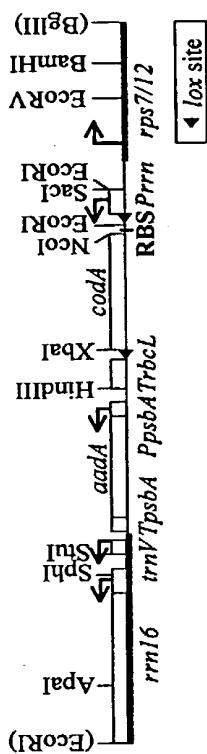


Figure 2

SUBSTITUTE SHEET (RULE 26)

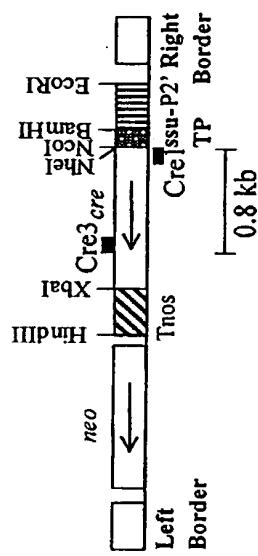


Figure 3

3/20

PCT/US00/25930

WO 01/21768

10/088634

4/20

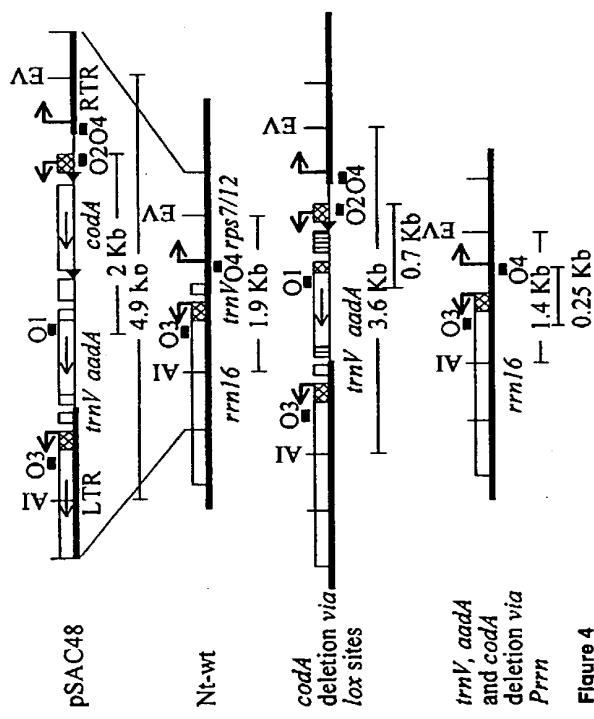


Figure 4

10/088634

PCT/US00/25930

WO 01/21768

5/20

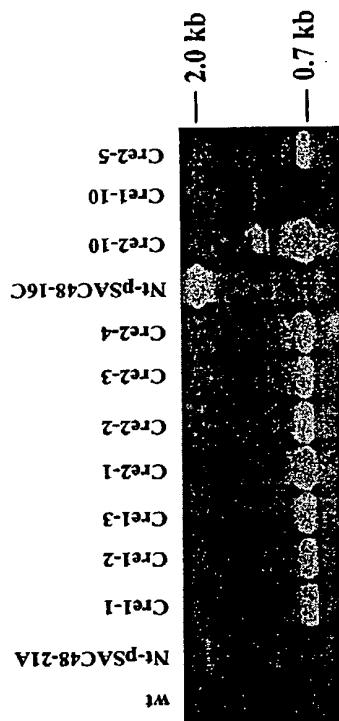


Figure 5

10/088634

WO 01/21768

PCT/US00/25930

6/20



Figure 6

10/088634

WO 01/21768

PCT/US00/25930

7/20

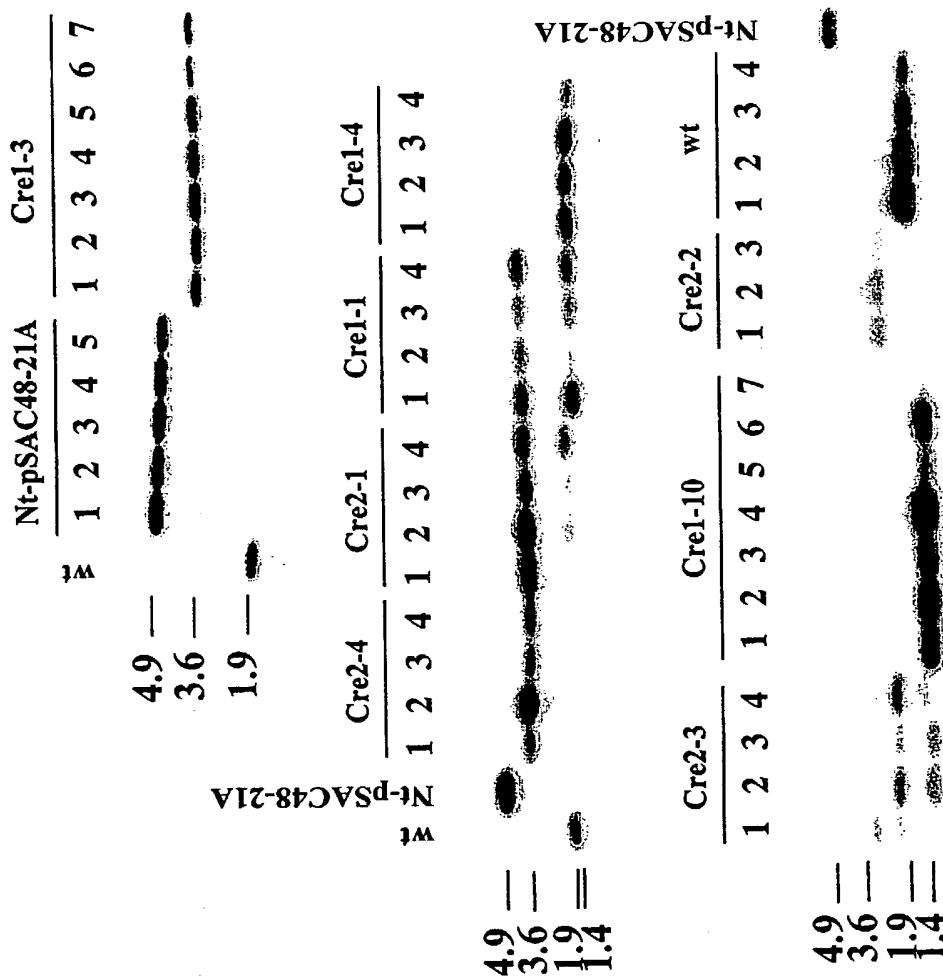


Figure 7

10/088634

PCT/US00/25930

WO 01/21768

8/20

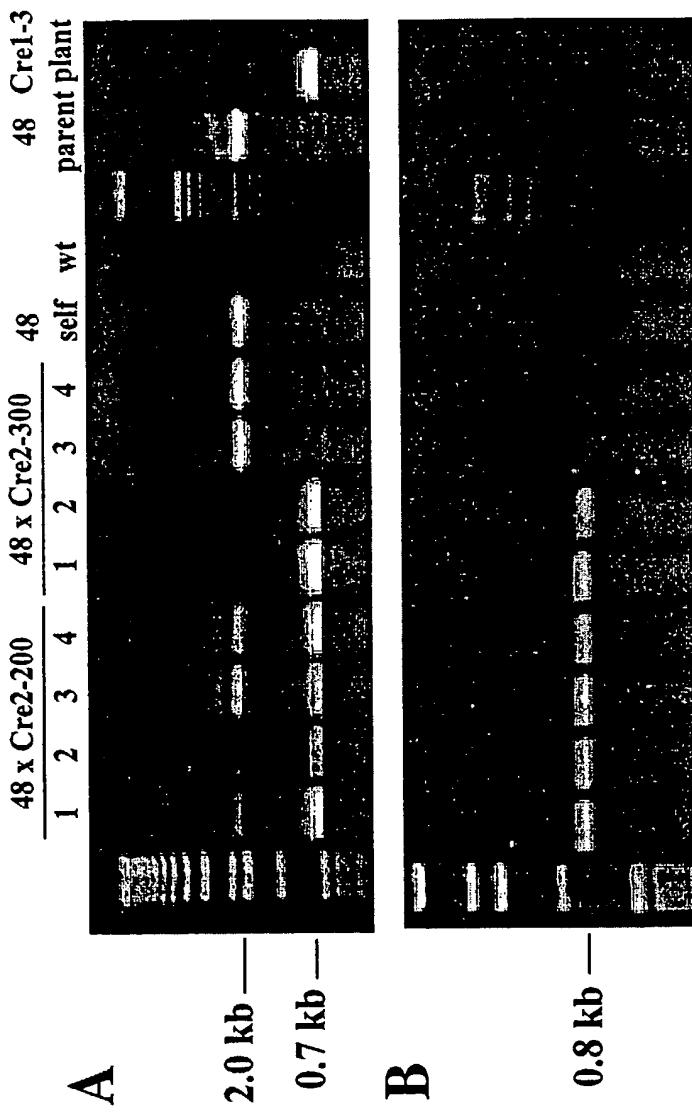
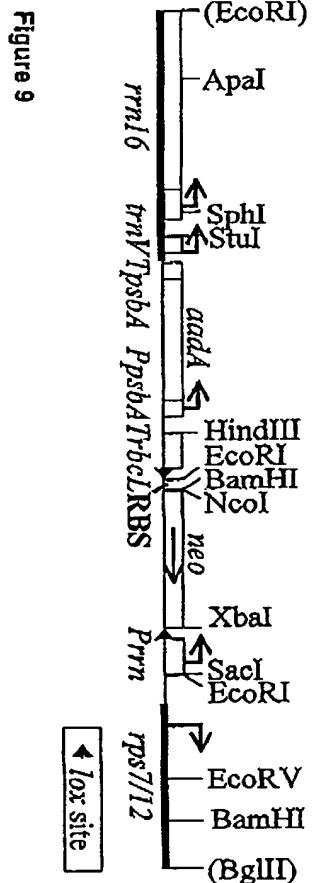


Figure 8



10/20

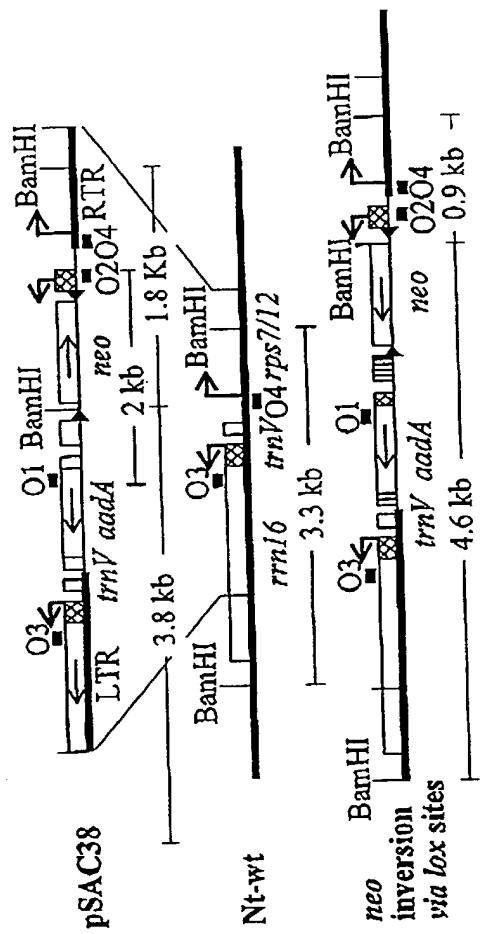
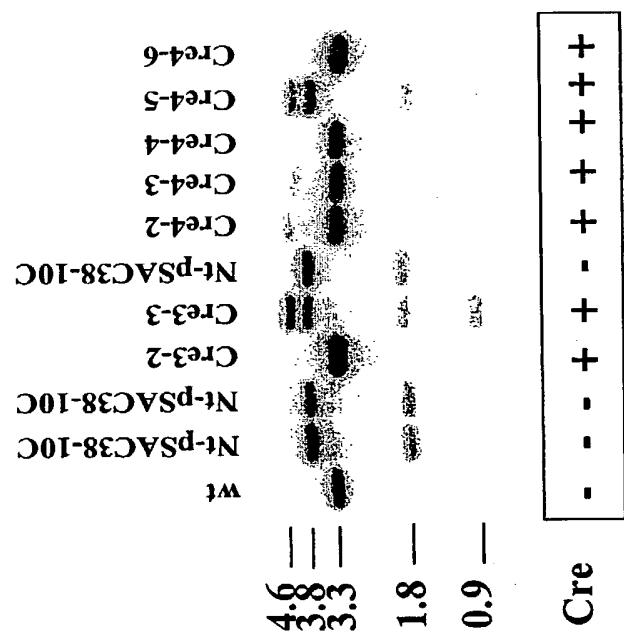


Figure 10

10/088634  
PCT/US00/25930

11/20



**Figure 11**

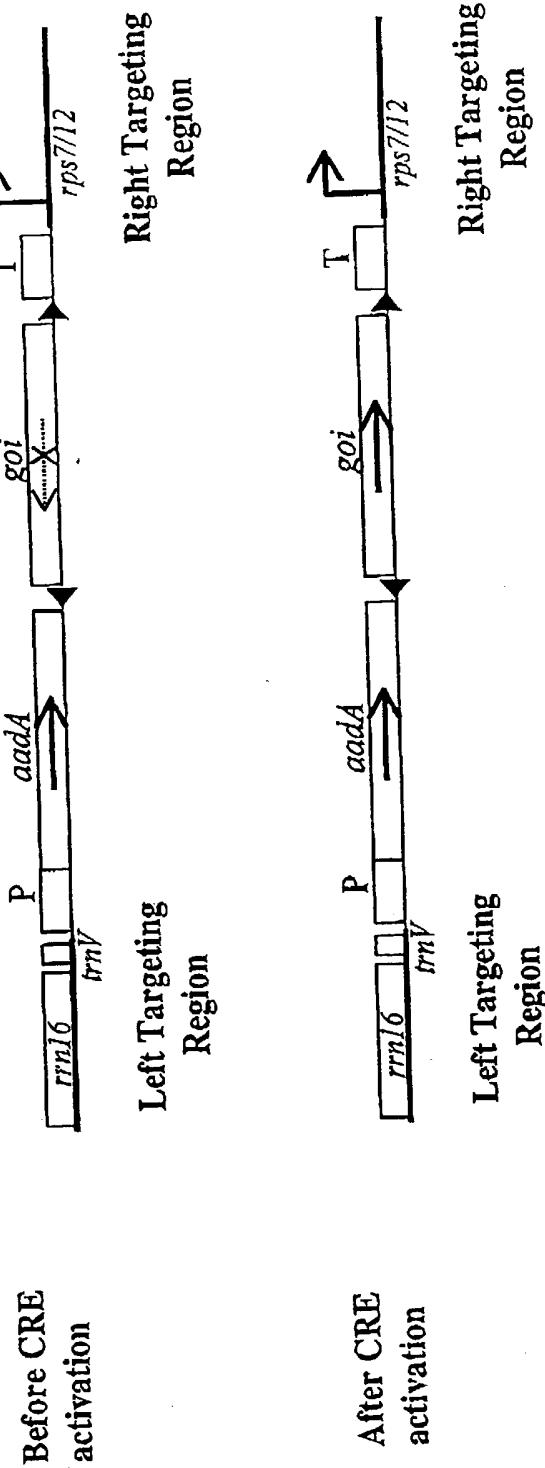


Figure 12

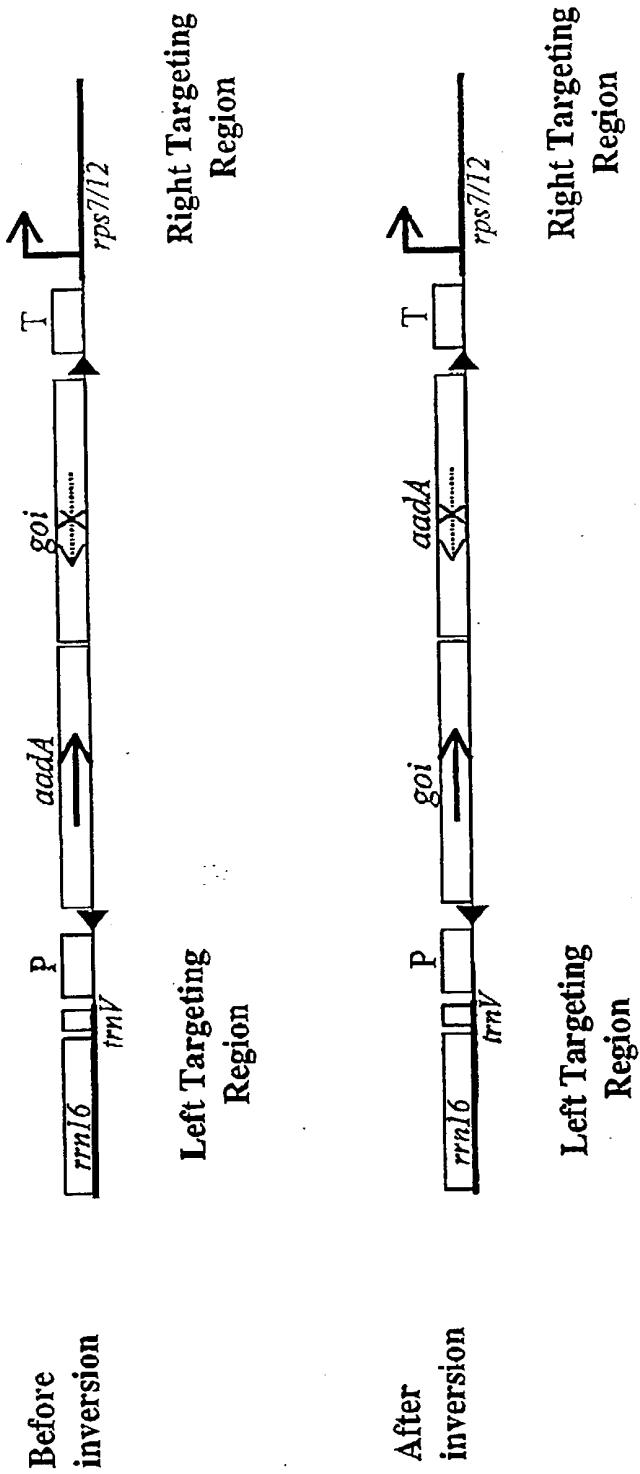


Figure 13

14/20

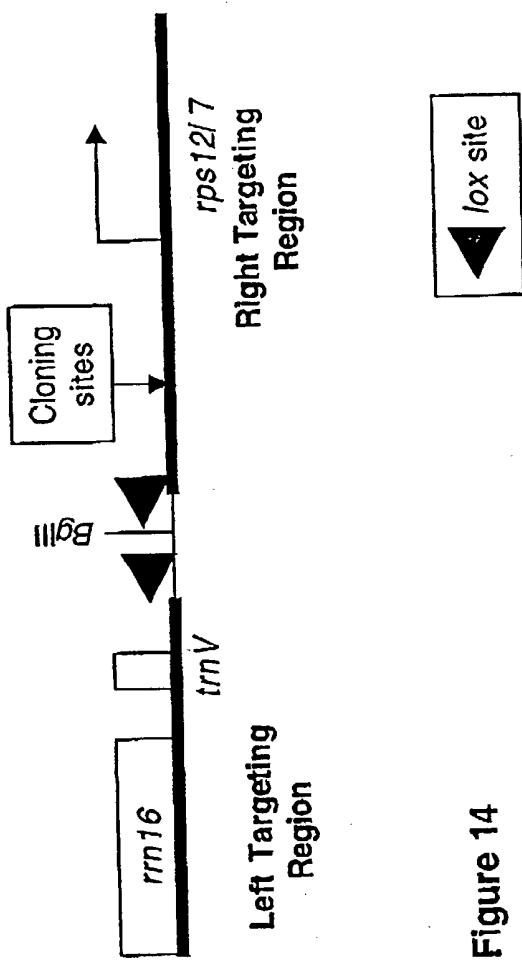


Figure 14

15/20

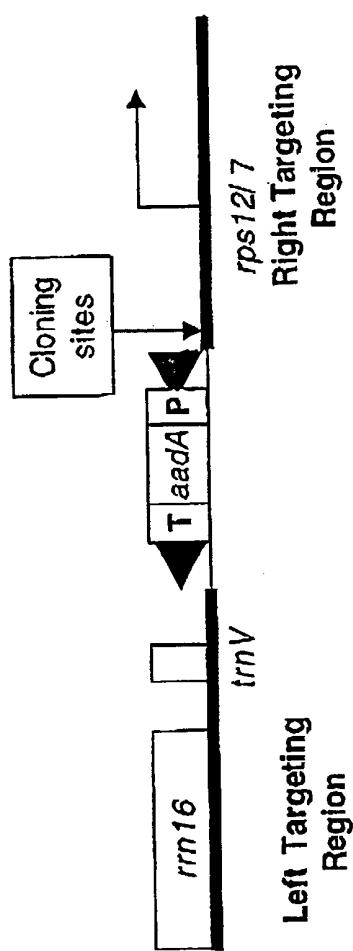


Figure 15

16/20

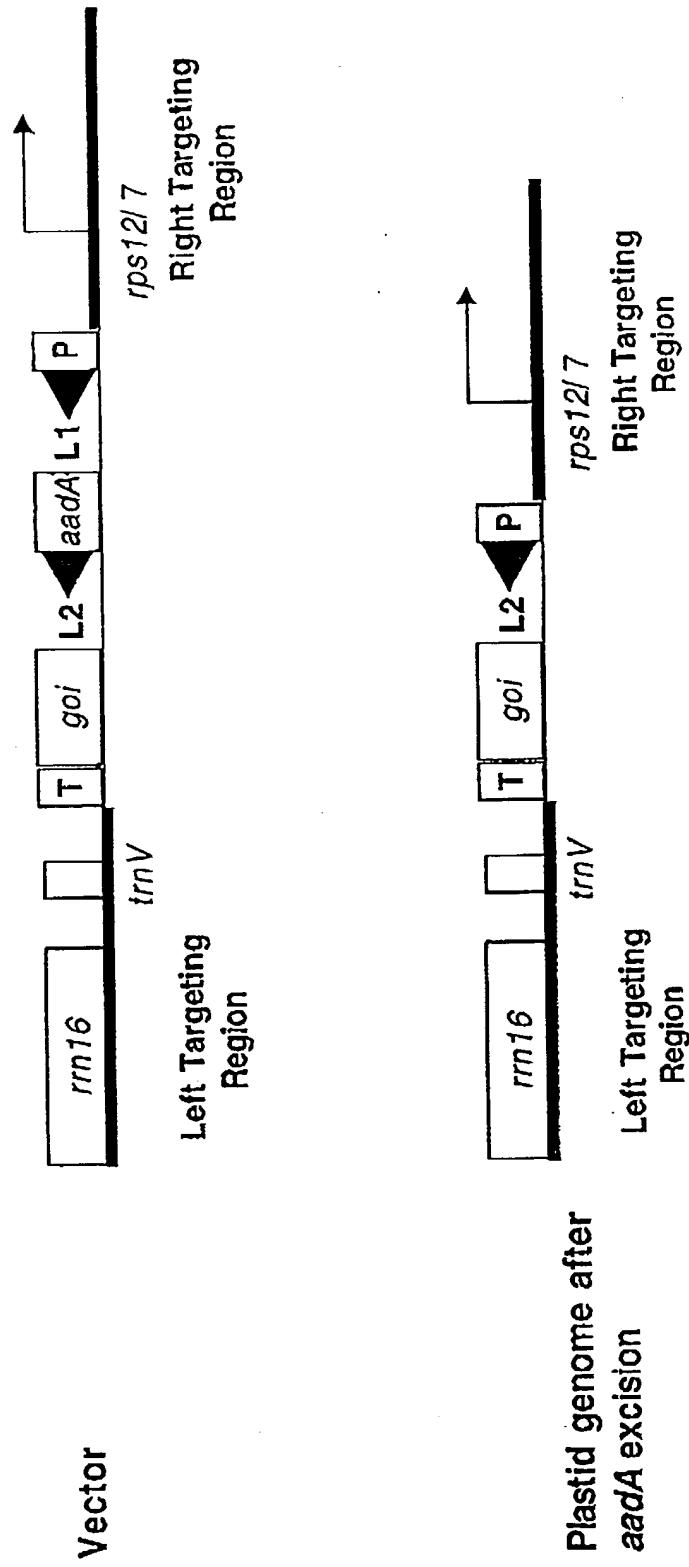


Figure 16

17/20

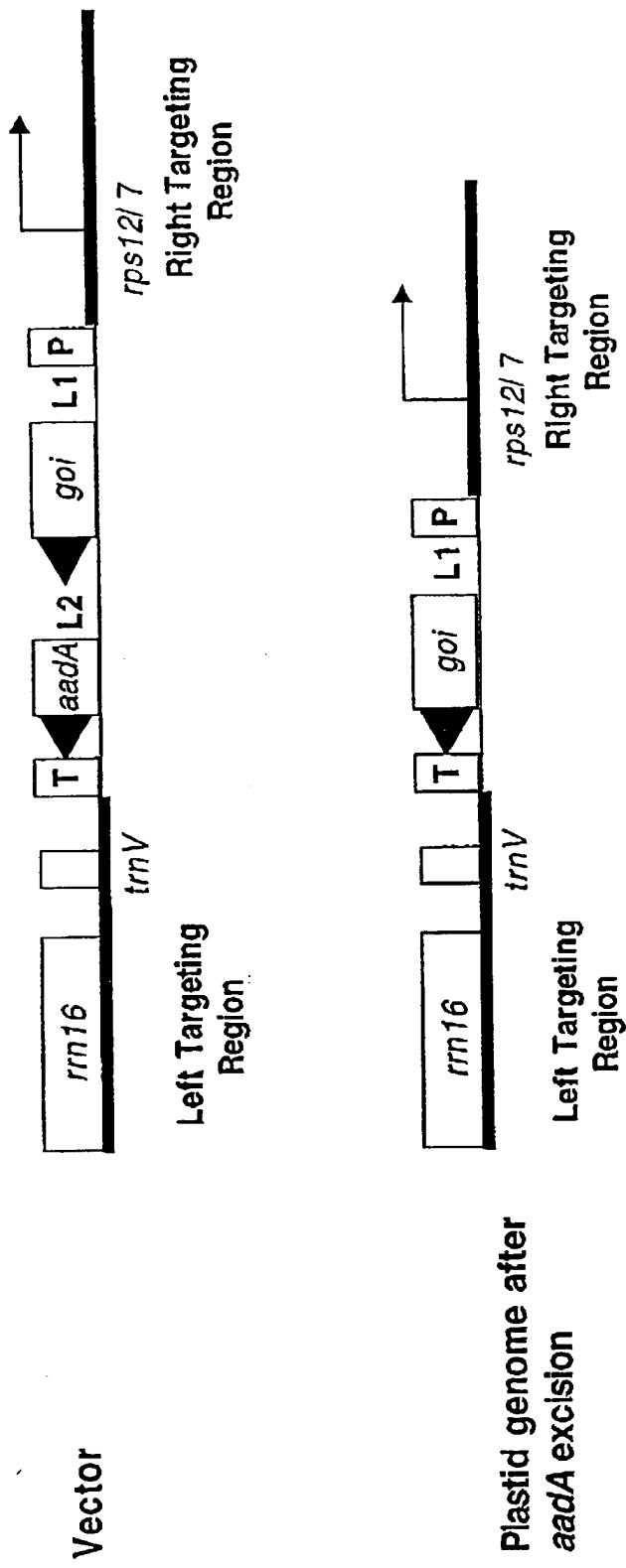


Figure 17

18/20

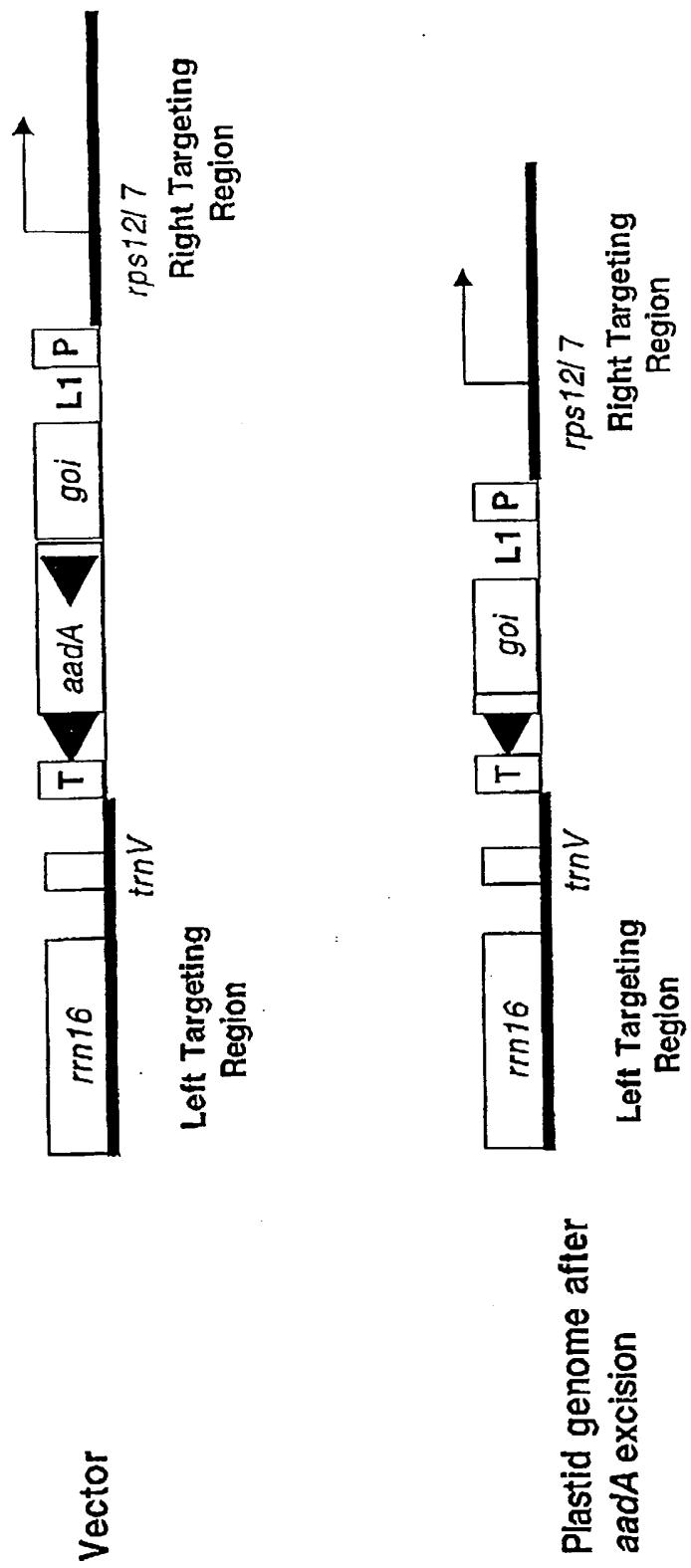


Figure 18

10/088634

PCT/US00/25930

WO 01/21768

19/20

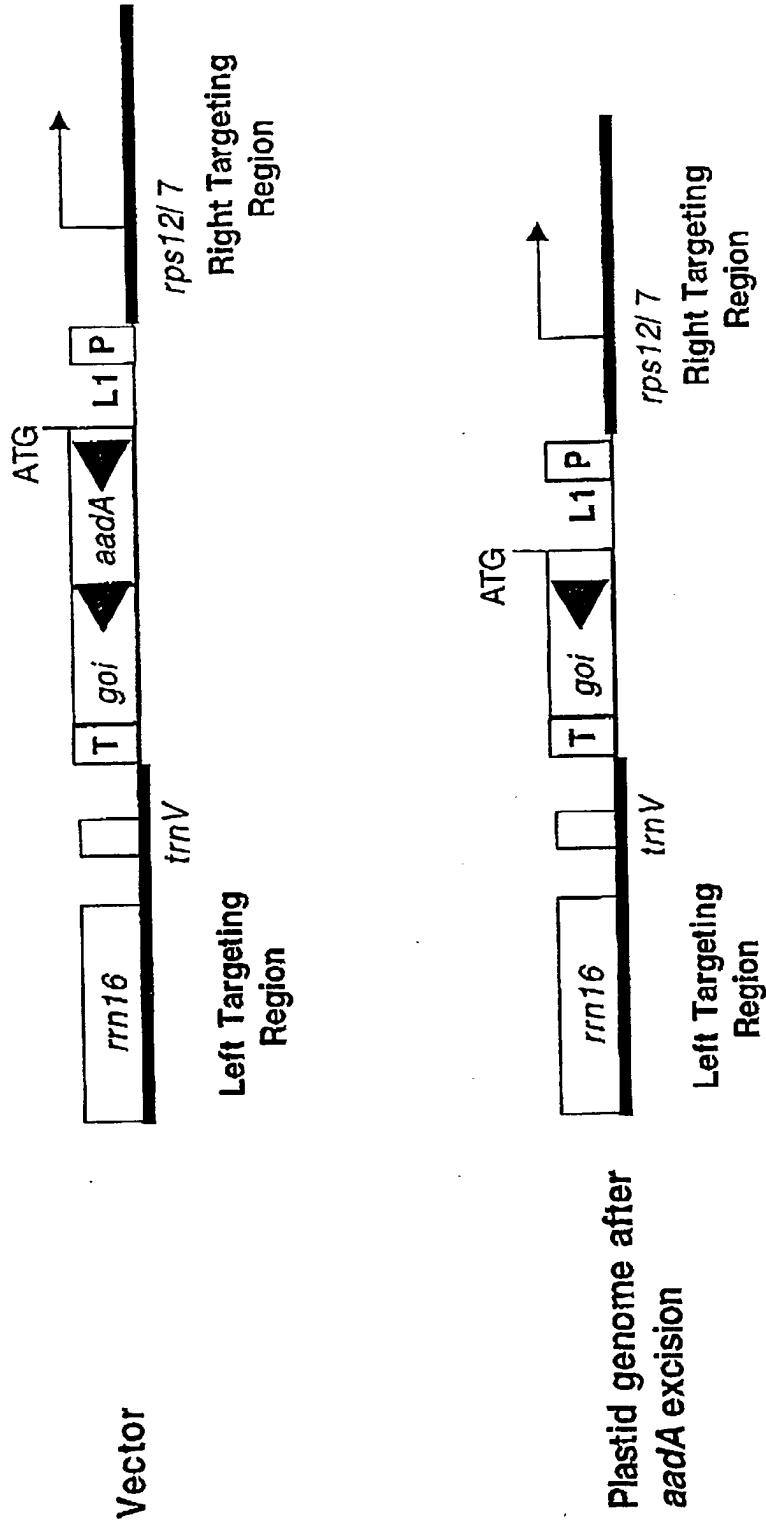


Figure 19

10/088634

PCT/US00/25930

WO 01/21768

20/20

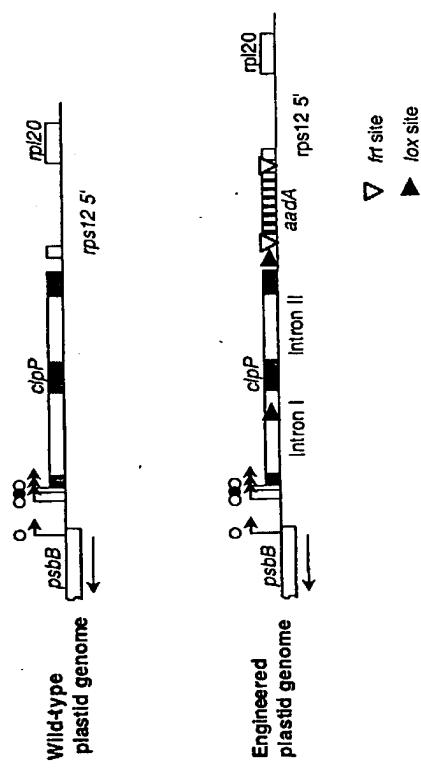


Figure 20

**DECLARATION, POWER OF ATTORNEY AND POWER TO INSPECT**

As a below named inventor, I hereby declare:

that my residence, post office address and citizenship are as stated below next to my name;

that I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention entitled: SITE-SPECIFIC RECOMBINATION SYSTEM TO MANIPULATE THE PLASTIC GENOME OF HIGHER PLANTS

the specification of which [check one(s) applicable]

was filed September 21, 2000 as U.S. International Patent Application No. PCT/US00/25930 on which U.S. Patent Application No. 10/088,634 file March 20, 2002 is based.

and was amended by Amendment filed \_\_\_\_\_ (if applicable); [or];

is attached to this Declaration, Power of Attorney and Power to Inspect;

that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and that I acknowledge my duty to disclose information which is material to the examination of this application in accordance with Rule 56 (a) (37 C.F.R. §1.56(a)).

**CLAIM UNDER 35 USC §119(e):** I hereby claim the benefit under 35 USC §119(e) of any United States provisional applications listed below:

<u>Provisional Application No.</u>	<u>Filing Date Day/Mo/Year</u>
60/155,007	21 September 1999
60/211,130	13 June 2000

**POWER OF ATTORNEY:** As inventor, I hereby appoint DANN, DORFMAN, HERRELL AND SKILLMAN, P.C. of Philadelphia, PA, and the following individual(s) as my attorneys or agents with full power of substitution to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Kathleen D. Rigaut, Ph.D., J.D. Reg. No. 43,047; Patrick J. Hagan, Esq. Reg. No. 27,643 and Maria Kouraklis, Esq. Reg. No. 41, 126

**POWER TO INSPECT:** I hereby give DANN, DORFMAN, HERRELL AND SKILLMAN, P.C. of Philadelphia, PA or its duly accredited representatives power to inspect and obtain copies of the papers on file relating to this application.

**SEND CORRESPONDENCE TO: CUSTOMER NUMBER 0000110.**

**DIRECT INQUIRIES TO:** Telephone: (215) 563-4100  
Facsimile: (215) 563-4044

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**SOLE OR FIRST JOINT INVENTOR**

Full Name Ted Maliga  
First Middle Last

Signature Ted Maliga

Date April 16, 2002 NJ

Residence East Brunswick New Jersey  
City State or Country

Citizenship United States of America  
Post Office Address:

72 Yorktown Road  
East Brunswick New Jersey 08816  
City State or Country Zip Code

**SECOND JOINT INVENTOR (IF ANY)**

Full Name Sylvie Cornelle  
First Middle Last

Signature Sylvie CORNELLE

Date April 18, 2002 NJ

Residence Highland Park, New Jersey  
City State or Country

Citizenship France  
Post Office Address:

120 Harper Street, Apt. 2B  
Highland Park, New Jersey 08904  
City State or Country Zip Code

U.S. Patent Application No. 10/088,634  
Page 2 of Declaration, Power of Attorney and  
Power to Inspect

THIRD OR FIRST JOINT INVENTOR

Full Name Kerry Intz  
First Middle Last

Signature Kerry Intz

Date 4/16/02 AS

Residence Lawrenceville New Jersey  
City State or Country

Citizenship United States of America  
Post Office Address:

100 Canal View Drive  
Lawrenceville New Jersey 08648  
City State or Country Zip Code

FOURTH JOINT INVENTOR (IF ANY)

Full Name \_\_\_\_\_  
First Middle Last

Signature \_\_\_\_\_

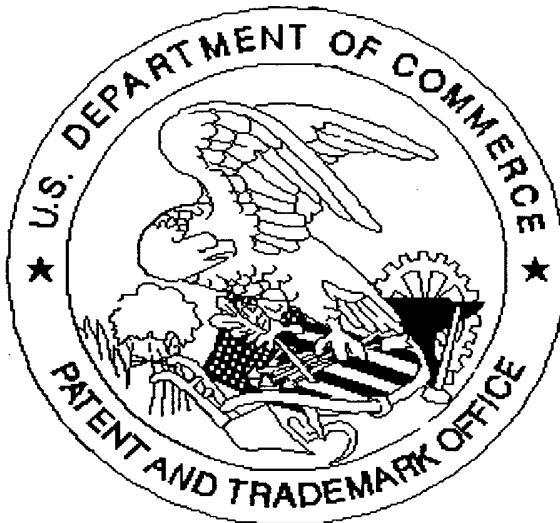
Date \_\_\_\_\_

Residence \_\_\_\_\_  
City State or Country

Citizenship \_\_\_\_\_  
Post Office Address:

City State or Country Zip Code

United States Patent & Trademark Office  
Office of Initial Patent Examination -- Scanning Division



Application deficiencies found during scanning:

Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not present  
for scanning. (Document title)

Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not  
present  
for scanning. (Document title)

*Scanned copy is best available. following fig 8 is dark*